Remarks

Applicants thank the Examiner for the telephone interview on January 27, 2004. The claims have been amended based on the Examiner's suggestions.

Claims 36, 39 and 41-49 are currently being examined. Claims 12-13, 16-35 and 50-56 have been canceled as being drawn to a non-elected invention. Claims 44 and 45 have also been canceled. Claims 35, 42, 43 and 47 have been amended and new claims 57-70 have been added. Support for the claim amendments and new claims may be found throughout the specification, including the claims as originally filed. In particular, support for the amendment to claim 36 can be found, e.g., at page 20, line 25 ("90% identical"); page 30, line 10 ("amino acids 148-192 of SEQ ID NO: 4"); and page 4, line 24 ("Skp1/Cull 1/F-box protein (SCF)"). Support for the amendment to claim 41 can be found, e.g., at page 12, line 23 ("beta TrCP" and "FWD1"). Support for the amendment to claim 42 ("95% identical") can be found, e.g., at page 20, line 25. Support for new claims 57-59 can be found, e.g., at page 30, line 10 ("amino acids 148-192 of SEQ ID NO: 4"). Support for new claim 58 can be found, e.g., at page 113, last line ("consists essentially of"). Support for new claim 60 can be found, e.g., at page 20, line 25 ("95% identical"). Support for new claims 60-61 can be found, e.g., at page 31, lines 2-7 (description of WD domains in SEQ ID NO: 4). Support for new claim 61 can be found, e.g., at page 113, last line ("consisting essentially of"). Support for new claims 65-67 can be found, e.g., at page 30, line 25 (90% identity" and "95% identity"). Support for new claims 69-70 can be found, e.g., at page 29, line 16 ("0.2 x SSC at 50° C"). No new matter has been added.

The Examiner notes that the species of SEQ ID NO: 4 is being considered. Should the elected species be patentable, Applicants are entitled to a search and examination of the remaining species covered by the claims. All the pending claims read on the elected species, and claims 36, 39, 41-43, 46-49, 62-63, 65-67, and 69-70 are generic claims encompassing the elected species.

Amendments and cancellations of claims should in no way be construed as an acquiescence to any of the Examiner's rejections. The amendments to and cancellations of claims are being made solely to expedite prosecution of the present application. Applicants reserve the option to further prosecute the same or similar claims in the instant or in a subsequent patent application.

Claim objections

Claim 36 and dependent claims 39 and 41-49 were rejected to allegedly because claim 36 recites "WD40 domain," whereas the specification recites "WD domain." Claim 36, as amended, does not recite "WD40 domain," however, to the extend that this objection applies to any of the new claims, Applicants respectfully traverse this objection. Indeed, the specification refers to both "WD domain" and "WD40 domain." See e.g., page 133, line 1.

Rejection of claim 44 under 35 U.S.C 112, first paragraph

Claim 44 stands rejected for reciting "F-box is at least 95% identical," whereas the specification allegedly does not provide adequate support for percent identity of 95%. Claim 44 has been canceled. However, to the extend that this rejection applies to any of the newly amended or added claims, Applicants respectfully traverse this rejection. "95%" can be found, e.g., at page 20, second line of the last paragraph.

Rejection of claims 36, 39 and 41-49 under 35 U.S.C 112, first paragraph

Claims 36, 39 and 41-49 stand rejected under 35 U.S.C. 112, first paragraph, for allegedly describing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the invention at the time the application was filed. The Examiner states that " 'F-box polypeptides that further comprise a WD domain' encompass proteins of diverse structures and, in many cases, unknown function" and that "[n]either 'F-box' nor 'WD40 domain' is defined by structure." Applicants respectfully traverse this rejection.

The claims are drawn to methods involving a hybrid polypeptide comprising an F-box from Cdc4 (SEQ ID NO: 2), hβTr CP (SEQ ID NO: 4), Grr1p (SEQ ID NO: 6), Met30p (SEQ ID NO: 8), pop2 (SEQ ID NO: 10), FWD1 (SEQ ID NO: 12) or a sequence that is homologous thereto, wherein the F-box recruits the hybrid polypeptide to a Skp1/Cul 1/F-box protein (SCF) ubiquitin ligase complex. In fact, it is the F-box of a protein that mediates the interaction of the protein with the Skp1 protein of the SCF ubiquitin ligase complex (see, e.g., Patton et al. (1998) *TIG* 14:236, attached hereto as Exhibit A, first sentence under the abstract). Skp1 proteins are highly conserved from one eukaryotic species to another (see, e.g., Fig. 1C of Bai et al., (1996) *Cell* 86:263, attached hereto as Exhibit B). Thus, F-boxes are highly conserved from one protein to another within the same species and across species. This is shown, e.g., in Fig. 3 of Patton et

al., *supra*, which shows an alignment of the F-box of 38 proteins from *S. cerevisiae* and Fig. 4A of Bai et al., *supra*, showing the homology of F-boxes across species.

Regarding WD (or WD40) domains, Applicants note that claims 36, 39, 41-43 and 47-49, as amended, do not recite "WD domain." WD domains are believed to mediate the interaction of F-box containing proteins with proteins that will be targeted for degradation (see, e.g., Patton et al., *supra*, sentence bridging pages 236 and 237 and page 242, under "Substrate recruitment domains in F-box proteins"). Accordingly, since the claimed hybrid proteins target proteins for degradation, which proteins do not normally interact with F-box containing polypeptides, WD domains are not necessary. In addition, it has indeed been shown that the WD domain is not necessary for targeting a target protein for ubiquitin dependent proteolysis with a hybrid protein, as claimed. For example, Su et al. (2003) *PNAS* 100:12729 (attached as Exhibit C) show that a hybrid protein comprising domains recognizing β-catenin (APCbc) linked to the F-box of βTrCP, and not containing any WD domain, results in targeted degradation of β-catenin (see, e.g., Fig. 1C and last sentence of the left column of page 12733). Similarly, Liu et al. (2004) *BBRC* 313:1023 (attached as Exhibit D) show that a hybrid protein containing the F-box of βTrCP, but not any of the WD domains, linked to the β-catenin-binding domains of Tcf4 and E-cadherin successfully promotes degradation of β-catenin (see, e.g., end of the Abstract). Other experiments performed by the inventors confirm that WD domains are not necessary.

Although the Examiner had pointed out in a previous office action that the specification allegedly indicates that the WD domain is necessary, and referred to page 133 of the specification, Applicants respectfully submit that the experiment described at page 133 describes that a protein (β-galactosidase) that is fused to Cdc4p failed to be targeted for degradation if the Cdc4p protein lacked WD repeats. In contrast, the claimed methods pertain to targeted degradation of a protein that interacts with a hybrid protein, but is not fused thereto. It is believed that in the case of the Cdc4p-β-galactosidase fusion protein of page 133, the degradation of β-galactosidase by a ubiquitin ligase complex may have required its proper positioning relative to Cdc4p for accepting ubiquitin from the ubiquitin-conjugating enzyme, which was provided by the presence of the WD domain. In effect, the WD domain in the Cdc4-β-galactosidase fusion protein is believed to merely have played the role of a linker between the F-box of Cdc4p and β-galactosidase.

Thus, since the claimed hybrid proteins have a common structure due to the presence of the F-box, a person of skill in the art would recognize that the inventors had possession of the invention at the time of filing. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Rejection of claims 36, 30 and 41-49 under 35 U.S.C 112, first paragraph

Claims 36, 39 and 41-49 stand rejected under 35 U.S.C. §112, first paragraph, allegedly as containing subject matter which was not described in the specification in such a way as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims. Specifically, the Examiner states that "while being enabling for a method of degradation of target polypeptides using hybrid polypeptides comprising Cdc4/βTrCP and [a] known target polypetide interaction domain, such as LTP and E7N, in yeast and human cells, respectively, does not reasonably provide enablement for a method of use of a hybrid comprising any F-box polypeptide for which target polypeptide and ubiquitin proteolysis pathway is unknown." Applicants respectfully traverse this rejection.

The specification describes that expressing a hybrid protein comprising an F-box and a target polypeptide interaction domain that binds to a target polypeptide in a cell results in targeted degradation of the target polypeptide. Indeed, without wanting to be limited to a particular mechanism of action, it is believed that the hybrid protein comprising an F-box recruits the target polypeptide interacting with the hybrid protein to the Skp1/Cul 1/F-box protein (SCF) ubiquitin ligase complex, to thereby target it for ubiquitin-dependent proteolysis. As set forth above, a WD domain is not necessary in this system. As also described above, F-boxes in different proteins are highly conserved and thus have a similar structure. Thus, due to this conservation, F-boxes of proteins from one eukaryotic species are believed to target proteins for degradation in cells of the same eukaryotic species, as well as cells from other eukaryotic species.

The specification further describes that target polypeptide interaction domains, if not known, can readily be isolated (see, e.g., pages 33 to 58). Among the recited methods are the yeast two-hybrid or interaction trap, the yeast cytoplasmic two-hybrid, the mammalian two-hybrid or interaction trap, the far western, phage display, protein trap + nucleic acid snag, biomolecular interaction analysis and peptide matrix arrays. Table I on page 36 provides a summary of the general methods, along with references, that may be used to clone interacting proteins and pages 37-58 provide additional guidance on these methods. Based on the guidance provided in the specification and the advanced level of the knowledge in the art, Applicants respectfully submit that a person skilled in the art would readily be able to identify and isolate a polypeptide interaction domain that could be used to construct a hybrid polypeptide of the present invention.

The specification provides working examples of fusion proteins comprising an F-box from two different proteins: pCdc4 and βTrCP, which as discussed in Applicants' previous response, are not the yeast and human homolog of one and the same protein. Regarding target proteins, the specification provides working examples of three different target proteins: pRb, p107 and the viral protein E2. In addition, since filing of the application, the Applicants have also successfully targeted other cellular proteins, e.g., p130, a retinoblastoma protein family member (Zhang et al. (2003) *PNAS* 100:14127, attached as Exhibit E) and β-catenin (Cong et al. (2003) *BMC Molecular Biology* 4:10, attached as Exhibit F).

Since the filing of the application, several articles have been published describing a similar system targeting a target protein for ubiquitin-mediated degradation by the SCF ubiquitin ligase complex: Liu et al., *supra*; and Su et al., *supra*, both of which describe the targeted degradation of β-catenin using different hybrid proteins. Accordingly, the specification enables a person of skill in the art to make and use the invention commensurate with the scope of the claims.

In applying the Wands factors, it is evident from the above discussion that the level of skill in this art was very high at the time the application was filed, and the experimental techniques needed to practice the invention were well known and exemplified in the specification as filed. Accordingly, Applicants respectfully submit that armed with the teachings of the specification and the contemporary knowledge in the art, the skilled artisan would be able to practice the claimed methods without further undue experimentation.

The Examiner's specific arguments are believed to be addressed in the above paragraphs, as well as in Applicants' previous responses. Thus, reconsideration and withdrawal of this rejection is respectfully requested.

Rejection of claim 45 under 35 U.S.C. 112, first paragraph

The Examiner additionally rejected claim 45 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, because "the specification, while being enabling for a hybrid protein comprising F-box polypeptide of SEQ ID NO:4, does not reasonably provide enablement for a hybrid protein comprising F-box polypeptide encoded by a DNA that hybridizes to SEQ ID NO: 3 under medium hybridization conditions comprising 0.2 x SSC at 50° C."

Claim 45 has been canceled, thereby rendering this rejection moot.

Rejection of claims 36, 39 and 41-49 under 35 U.S.C. 112, second paragraph

Claims 36, 39 and 41-49 stand rejected under 35 U.S.C. 112, second paragraph, allegedly as being indefinite. The claims have been amended and are believed to overcome the rejections. Thus reconsideration and withdrawal of the rejection is respectfully requested.

Rejection of claims 36-39, 45, 46, 48 and 49 under 35 U.S.C. 102(b)

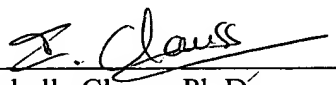
Claims 36, 39, 46, 48 and 49 stand rejected under 35 U.S.C. 102(b) allegedly as being anticipated by Scheffner et al. (*Virology* 199: 448-452 (1994), form PTO-1449 mailed January 28, 2002 reference BF). The Examiner states that Scheffner et al. "teach degradation of the retinoblastoma protein by human papilloma virus type 16 (HPV-16) E7-E6 proteins in vitro" and that "absent clear definition of 'F-box polypeptide,' E-6 is construed as an F-box polypeptide." Applicants respectfully traverse the rejection.

The claims have been amended, and as such are not believed to be anticipated by Scheffner et al. Reconsideration and withdrawal of this rejection is respectfully requested.

Conclusion

In view of the above remarks and the amendments to the claims, it is believed that this application is in condition for allowance. If a telephone conversation with Applicant's Attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 832-1000.

Respectfully submitted,

By: 
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Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis

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The ubiquitin-dependent proteolytic pathway targets many key regulatory proteins for rapid intracellular degradation. Specificity in protein ubiquitination derives from E3 ubiquitin protein ligases, which recognize substrate proteins. Recently, analysis of the E3s that regulate cell division has revealed common themes in structure and function. One particularly versatile class of E3s, referred to as Skp1p–Cdc53p–F-box protein (SCF) complexes, utilizes substrate-specific adaptor subunits called F-box proteins to recruit various substrates to a core ubiquitination complex. A vast array of F-box proteins have been revealed by genome sequencing projects, and the early returns from genetic analysis in several organisms promise that F-box proteins will participate in the regulation of many processes, including cell division, transcription, signal transduction and development.

Many cellular processes are regulated by a diverse array of proteolytic mechanisms. Salient examples include the protease cascades in apoptosis, lysosomal/endosomal pathways in receptor degradation and secreted proteases in remodeling of the extracellular matrix (see a recent special issue of *Trends in Biochemical Sciences* for further reading[1]). However, it is the ubiquitin-dependent proteolytic pathway that has emerged as the principal arbiter of the fate of a protein inside the cell[2]. Ubiquitin is a highly conserved 76 amino acid protein that tags substrate proteins for degradation by an abundant 26S protease complex called the proteasome[3]. As defined by the seminal work of Hershko *et al.*[4], ubiquitin is activated in a thioester linkage to a ubiquitin activating enzyme (E1), then transferred to a ubiquitin conjugating enzyme (E2) and, in conjunction with a ubiquitin protein ligase (E3), ultimately conjugated to a lysine residue of a substrate protein in an isopeptide linkage. Reiteration of this cascade rapidly assembles a polyubiquitin chain on the substrate, which targets it for capture and degradation by the proteasome. The very short halflife of many proteins degraded by the ubiquitin-proteasome pathway[2], as well as recent experiments showing that access of proteasomes to most of the intracellular volume is essentially diffusion limited[5], underscore the ruthless efficiency of this system.

E3 ubiquitin-protein ligases mediate the critical step of substrate recognition. Until recently, it has been difficult to identify E3s because different E3 families share little or no sequence similarity and also because E3s are often low abundance, rate-limiting components in protein ubiquitination. Because E3s are so varied, a precise definition of an E3 is somewhat elusive. At minimum, an E3 must interact with the substrate and its cognate E2, although in some cases the E3 also forms a catalytic intermediate with ubiquitin[2]. Although many E3s are single proteins, recent evidence suggests that multiprotein E3 complexes are prevalent. Intense efforts in the cell-cycle field have uncovered two classes of evolutionarily conserved E3 complexes, the Skp1p–Cdc53p (cullin)–F-box protein (SCF) complexes and the anaphase-promoting complex (APC; also called the cyclosome), which are required for the G1–S phase transition and the metaphase–anaphase transition, respectively (Fig. 1)[6]. In this review, we describe recent insights into these E3 complexes, and the possible relevance of these insights to other cellular pathways.

The F-box hypothesis

Analysis of several cell division cycle (*cdc*) mutants in yeast provided the inroad into the role of ubiquitin-dependent proteolysis in the G1–S phase transition. In the budding yeast *Saccharomyces cerevisiae*, loss of Cdc4p, Cdc34p or Cdc53p function causes cells to arrest in G1 phase because of a failure to degrade Sic1p, a potent inhibitor of the CLB–Cdc28p kinases, which activate DNA replication (Fig. 1)[7]. Similarly, Cdc34p, Cdc53p and another protein, Grr1p, are required for degradation of the G1 cyclins, Cln1p and Cln2p (Refs 8–10). It became apparent that multiprotein complexes lie behind the genetics when it was found that Cdc34p, an E2 enzyme, is physically associated with Cdc4p and Cdc53p (Refs 10, 11). The full elaboration of these degradation pathways came with the identification of a protein called Skp1p and its binding site, the F-box motif[12]. Human SKP1 was originally isolated in conjunction with its binding partner SKP2 as part of a cyclin A–CDK2 complex[13], and subsequently as a cyclin F-interacting protein[12]. In parallel, the yeast Skp1p homolog was isolated as a high copy suppressor of both the *cdc4-1* and the *ctf13-30* mutations[12,14], and as part of the CBF3 kinetochore complex (see below)[15]. Some conditional alleles of *skp1* result in stabilization of Sic1p, which causes a cell-cycle arrest with a *cdc4*-like phenotype, while other alleles cause stabilization of Cln2p and cell-cycle arrest in G2 phase[12,14]. In a key insight, Elledge and co-workers aligned the Skp1p interacting proteins and deduced the Skp1p binding site, a degenerate 40 amino acid motif called the F-box, after cyclin F (Ref. 12). Subsequent inspection showed that Grr1p also contains an F-box. Because the Sic1p and Cln1p, -2p degradation pathways appeared to require the same core components (i.e. Cdc34p, Cdc53p and Skp1p) but different F-box proteins (i.e. Cdc4p versus Grr1p), it was argued that Skp1p serves in multiple degradation pathways by virtue of its ability to assemble complexes with different F-box proteins, which recruit specific substrates for degradation[12]. This idea is sometimes referred to as the 'F-box hypothesis'. One tenet of the F-box hypothesis is that protein–protein interaction domains in F-box proteins, such as the WD40 repeats[16]

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present in Cdc4p and the leucine-rich repeats (LRRs)[17] present in Grr1p, recruit substrates for ubiquitination[12]. A second tenet is that Skp1p targets many proteins for ubiquitination through the myriad F-box proteins that can be identified in sequence databases[12]. Recent studies have validated the F-box hypothesis, but not without some interesting twists.

SCF pathways in yeast

The protein–protein interactions that underlie F-box protein-mediated ubiquitination are now in place (Fig. 2). In addition to binding Cdc4p and other F-box proteins, Skp1p also binds directly to an N-terminal region of Cdc53p, thereby linking Cdc53p to various F-box proteins[18–20]. These Skp1p–Cdc53p–F-box protein complexes are designated SCF, for example, SCF^{Cdc4} or SCF^{Grr1}. Because Cdc53p homologs in metazoans are called cullins[21], this nomenclature also accommodates the Skp1p–cullin–F-box protein complexes found in other species[22]. Within the SCF complex, Cdc53p provides an independent binding site for Cdc34p, and thus acts as a scaffold protein for the Cdc34p–Cdc53p–Skp1p core E2–E3 ubiquitination complex[18–20]. SCF complexes therefore fulfill the minimal criteria for an E3, namely the ability to juxtapose substrates and an E2 enzyme. Unlike some E3s, there is no evidence that SCF complexes form ubiquitin thioester intermediates[20].

SCF^{Cdc4}

The mechanism of Sic1p degradation is now understood in considerable detail, and has largely conformed to models based initially on genetic data[7,23,24]. As yeast cells approach the G1–S phase transition, activity of the CLN–Cdc28p kinases mounts, resulting in Sic1p phosphorylation, and recruitment of phospho-Sic1p into the SCF^{Cdc4} complex, where it is ubiquitinated (Fig. 1). In support of this scheme, mutation of the multiple CLN–Cdc28p phosphorylation sites in Sic1p stabilizes Sic1p *in vivo*, and overproduction of such mutants phenocopies the *cdc4* arrest (Ref. 25; M. Mendenhall, pers. commun.). Recent successes in reconstitution of SCF^{Cdc4} complexes from recombinant proteins have demonstrated that the components identified by genetics are necessary and sufficient for ubiquitination of Sic1p *in vitro*[18,19]. As predicted by the F-box hypothesis, recruitment of phospho-Sic1p into the SCF^{Cdc4} complex requires the WD40 repeat domain of Cdc4p (Ref. 18). The SCF^{Cdc4} complex is thus an excellent template to which other SCF complexes can be compared.

Although degradation of Sic1p is the essential G1 function of SCF^{Cdc4}, other proteins are targeted by this E3 complex (Table 1). A second CDK inhibitor, Far1p, is another key substrate of SCF^{Cdc4}. Far1p inhibits CLN–Cdc28p kinases in cells arrested by mating pheromone and, in a delicate regulatory balance, the CLN–Cdc28p kinases phosphorylate Far1p and target it for degradation by SCF^{Cdc4} (Ref. 26). Far1p can be ubiquitinated *in vitro* in a phosphorylation-dependent manner, and akin to Sic1p mutants, overexpression of a phosphorylation site mutant of Far1p causes pheromone-independent G1 arrest[26]. The replication activator Cdc6p is also targeted to SCF^{Cdc4} by CDK-dependent phosphorylation; however, in this case the physiological relevance of Cdc6p degradation is unclear because mutational stabilization of Cdc6p causes no obvious phenotype[27]. Finally, SCF^{Cdc4} probably mediates phosphorylation-dependent degradation of Gcn4p, a transcription factor required for induction of many amino acid biosynthesis genes (Ref. 28; D. Kornitzer, pers. commun.). An obvious theme among the multiple substrates of the SCF^{Cdc4} pathway is phosphorylation-dependent recognition, but as yet a consensus recognition site has not emerged from the substrates.

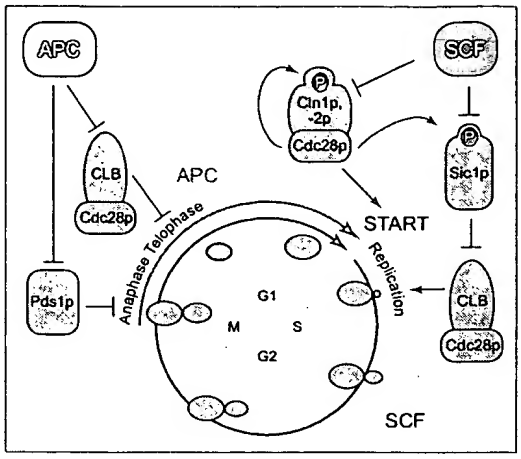

FIGURE 1. Interplay between cyclin-dependent kinases (CDKs) and ubiquitin-dependent proteolysis in cell-cycle regulation. The two major cell-cycle transitions in budding yeast, START and mitosis, require the activation of the CDK Cdc28p by G1 cyclins (Cln1p, -2p, -3p) and mitotic cyclins (Clb1p, -2p, -3p, -4p, -5p, -6p), respectively. At START, Cln1p, -2p–Cdc28p phosphorylates Sic1p and thereby targets it to a Skp1p–Cdc53p–F-box protein^{Cdc4} (SCF^{Cdc4}) complex for ubiquitination and degradation. Similarly, Cln1p, -2p–Cdc28p autophosphorylation targets Cln1p, -2p to the SCF^{Grr1} pathway. Once Sic1p is eliminated, the Clb5p, -6p–Cdc28p kinases activate DNA replication and after completion of replication the Clb1p, -2p, -3p, -4p–Cdc28p kinases initiate entry into mitosis. The metaphase to anaphase transition requires the anaphase-promoting complex (APC), which degrades the anaphase inhibitor Pds1p, the CLBs and other proteins. SCF pathways appear to be constitutively active throughout the cell cycle (purple), whereas the APC is active from anaphase until the end of G1 phase (green). Red arrows indicate phosphorylation; black arrows indicate activation; lines ending with bars indicate inhibition.

The control of DNA replication by the SCF^{Cdc4} pathway is conserved through evolution. In *Schizosaccharomyces pombe*, two Cdc4p homologs, Pop1 and Pop2, regulate DNA replication through phosphorylation-dependent degradation of Rum1 and Cdc18, which have analogous functions to Sic1p and Cdc6p, respectively (Ref. 29; T. Toda, pers. commun.). Deletion of *pop1* or overexpression of phosphorylation site mutants of either Rum1 or Cdc18 causes DNA re-replication[29–33]. Homologs of Skp1p and Cdc53p are also present in the *S. pombe* genome, but their function has yet to be assigned. It seems likely that the SCF^{Cdc4} pathway will be conserved in vertebrates because function of a Cdc34p homolog is required for initiation of DNA synthesis in *Xenopus* extracts[34]. Finally, a SKP1–CUL1–SKP2–CDC34 complex has been detected in association with cyclin A–CDK2 in human cells (Ref. 35).

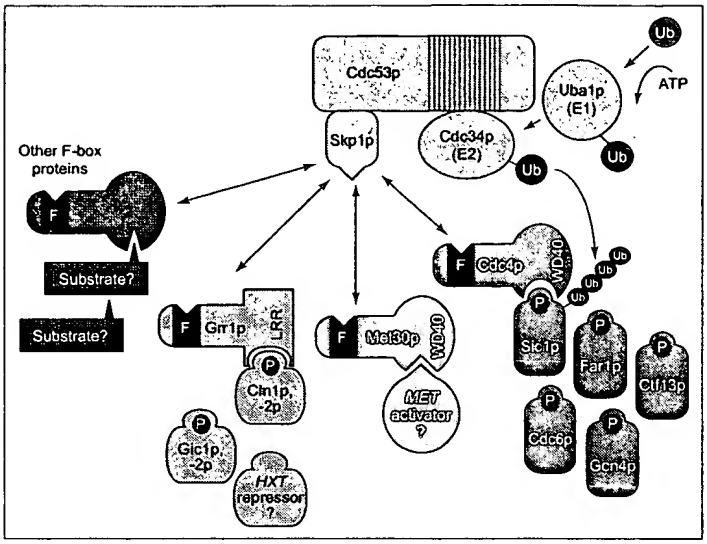

FIGURE 2. F-box proteins serve as adaptors in Skp1p–Cdc53p–F-box protein (SCF) complexes. Cdc34p, Cdc53p and Skp1p form a core E2–E3 ubiquitination complex that targets a variety of substrates through substrate specific F-box proteins (F indicates the F-box). Recognition of many substrates is phosphorylation-dependent (indicated by the black circle labeled P). In the standard SCF configuration, Cdc53p bridges Skp1p–F-box protein subcomplexes to the E2 enzyme Cdc34p, which provides the enzymatic activity for substrate ubiquitination (Ub). Polyubiquitinated substrates are recognized and degraded by the 26S proteasome. The Cdc34p binding site in Cdc53p maps to a broad region of similarity conserved between all Cdc53p homologs (indicated by vertical lines), whereas the Skp1p binding site resides in the poorly conserved N-terminus of Cdc53p (Ref. 20). See text for details.

SCF^{Grr1}

As originally anticipated, Grr1p forms an SCF complex with Cdc53p and Skp1p *in vivo* and *in vitro*[20,36] and *in vitro*[18]. CLN–Cdc28p kinase activity is inherently unstable *in vivo* because autophosphorylation of the CLN subunit within the CLN–Cdc28p kinase complex targets the CLN for SCF^{Grr1}-dependent degradation (Fig. 1)[8,10,37]. The *in vivo* specificity of F-box proteins for their substrates has been recapitulated *in vitro* with recombinant SCF^{Grr1} complexes, which specifically bind to phospho-Cln1p, -2p but not phospho-Sic1p (Ref. 18). *In vitro* ubiquitination of CLNs has not yet been achieved, perhaps because additional components or modifications are required for SCF^{Grr1} activity. Interestingly, Cln3p degradation does not depend on Grr1p (Ref. 9) but does appear to depend on Cdc34p and Cdc53p (Refs 10, 38), leading one to wonder whether yet another F-box protein targets Cln3p for ubiquitination.

Like Cdc4p, Grr1p probably recruits several substrates for ubiquitination (Table 1). Two proteins that help to initiate polarized bud growth, called Gic1p and Gic2p (Refs 39, 40), are strongly stabilized in *grr1* mutants, and their degradation depends on CLN–Cdc28p activity (M. Jaquenoud and M. Peter, pers. commun.). Grr1p is also required for the induction of *HXT* glucose transporter genes by glucose and, as predicted by the F-box hypothesis, *Skp1* and *Cdc53* mutants have a severe defect in *HXT1* induction[36]. The target of SCF^{Grr1} in this pathway is unknown, but it is inferred to be a transcriptional repressor of the *HXT* genes. Because *grr1* mutants have additional phenotypes, such as defects in cation and amino acid transport, it is possible that SCF^{Grr1} has other targets that might regulate expression of other transporter genes[36].

Despite the similarities, *HXT* regulation by SCF^{Grr1} is not precisely analogous to the SCF^{Cdc4} pathway. First, *HXT* induction occurs normally in a *Cdc34-2* strain[36]. This surprising finding might indicate that SCF^{Grr1} specifically regulates *HXT* gene expression in conjunction with another E2 (or that an E2 is not required!), or it might simply reflect allele specificity of *Cdc34* mutations. It is important to bear in mind that all alleles of SCF^{Cdc4} components were derived by selection for the CDC$^-$ phenotype caused by Sic1p stabilization, and so genetic results in other SCF pathways obtained with these alleles must be interpreted with caution. A second variation in the SCF^{Grr1} pathway is that unlike the Skp1p–Cdc4p interaction, which is mediated primarily by the F-box, the Skp1p–Grr1p interaction apparently requires both the F-box and the LRRs of Grr1p (Ref. 36). Thus the protein–protein interactions within SCF complexes might not always adhere to the strictly modular scheme depicted in Fig. 2. Interestingly, the Skp1p–Grr1p interaction is stimulated by glucose, the first indication that SCF complexes can be regulated at the level of assembly[36].

SCF^{Met30}

An F-box protein called Met30p mediates transcriptional repression of various *MET* genes by methionine[41]. Met30p associates with Skp1p and Cdc53p in an F-box-dependent manner, and in accord with the F-box hypothesis, Cdc34p, Cdc53p and Skp1p function is required for repression of *MET* genes[20]. The regulation of methionine biosynthesis genes by SCF^{Met30} is conserved in fungi because two Met30p homologs, Scon2 and SconB, regulate methionine biosynthesis in *Neurospora* and *Aspergillus*, respectively[42,43]. In addition, a Skp1p homolog called SconC is required for sulfur metabolism in *Aspergillus*[42]. Although an initial alignment of the three Met30p homologs revealed a conserved sequence that encompassed the F-box, at the time no function was assigned to this region[43]. The presumed target of SCF^{Met30} has yet to be identified but components of the Met4p transcription factor complex that activates *MET* gene expression are logical candidates[44]. Given that SCF^{Cdc4} and SCF^{Grr1} both have multiple targets, it would not be surprising if SCF^{Met30} also regulated other processes.

From the analysis of SCF^{Cdc4}, SCF^{Grr1} and SCF^{Met30}, it is clear that the core SCF components Cdc34p, Skp1p and Cdc53p are pleiotropic regulators. In contrast, remarkably little cross talk occurs between different F-box

TABLE 1. SCF complexes and their kin

F-box protein	Skp1 homolog	Cdc53/ cullin	E2	Other associated proteins	Probable targets	Target function or pathway	Refs
S. cerevisiae							
Cdc4p	Skp1p	Cdc53p	Cdc34p	–	Sic1p	CLB–Cdc28p inhibitor	7, 11, 12, 18–20, 25
Cdc4p	Skp1p	Cdc53p	Cdc34p	?	Far1p	CLN–Cdc28p inhibitor	26
Cdc4p	?	Cdc53p	Cdc34p	?	Cdc6p	Replication factor	27
Cdc4p	?	?	Cdc34p	?	Ctf13p	Kinetochore component	45
Cdc4p	Skp1p	Cdc53p	Cdc34p	?	Gcn4p	Transcription factor	28, a
Grr1p	Skp1p	Cdc53p	Cdc34p	?	Cln1p, -2p	G1 cyclins	8–10, 12, 18, 20, 37
Grr1p	Skp1p	Cdc53p	Cdc34p	?	Gic1p, -2p	Polarized bud growth	39, 40, b
Grr1p	Skp1p	Cdc53p	?	?	TXN repressor?	Glucose induction of *HXT* genes	20, 36
?	?	Cdc53p	Cdc34p	?	Cln3p	G1 cyclin	10, 38
Met30p	Skp1p	Cdc53p	Cdc34p	?	TXN activator?	Methionine repression of *MET* genes	20, 41
Ctf13p	Skp1p	–	–	Ndc10p Cep3p	CDEIII element	CBF3 kinetochore complex	14, 15, 45
S. pombe							
Pop1, -2	?	?	?	?	Rum1	CyclinB–Cdc2 inhibitor	29, 30, 33, c
Pop1, -2	?	?	?	?	Cdc18	Replication factor	29, 31, 32, c
N. crassa							
Scon2	?	?	?	?	?	Sulfur metabolism	43
A. nidulans							
SconB	SconC	?	?	?	?	Sulfur metabolism	42
A. majus							
FIM	FAP1, -2, -3	?	?	?	?	Floral organ development	59
A. thaliana							
UFO	?	?	?	?	?	Floral organ development	60
TIR1	?	?	?	?	?	Auxin response	61
C. elegans							
?	?	CUL-1	?	?	?	Activator of cell division	21
SEL-10	?	?	?	?	LIN-12/ Notch	AC/VU lateral fate decision	62
D. melanogaster							
Slimb	?	?	?	?	Cubitus interruptus	Transcription factor in HH pathway	63
Slimb	?	?	?	?	Armidillo	Transcription factor in WG pathway	63
H. sapiens							
Cyclin F	SKP1	?	?	?	?	G2 regulation?	12
SKP2	SKP1	CUL1	CDC34	CyclinA–CDK2	?	S-phase progression	13, 35
?	Elongin C	CUL2	?	VHL Elongin B	?	Cell-cycle exit; repression of hypoxia-induced mRNAs	48–50
Elongin A	Elongin C	–	–	Elongin B	?	Pol II transcription elongation	47
βTrCP	SKP1	?	?	VPU	CD4	CD4 degradation in HIV infected cells	65

Abbreviations: –, not identified in purified active complexes; ?, not determined.
[a]D. Kornitzer, pers. commun.
[b]M. Jaquenoud and M. Peters, pers. commun.
[c]T. Toda, pers. commun.

proteins *in vivo*[20]. Genetic analysis suggests that Cdc4p, Grr1p and Met30p (and probably other F-box proteins) compete for a limiting amount of the core Cdc34p–Cdc53p–Skp1p complex[11,20,36]. Thus, yeast has set the paradigm for combinatorial control of SCF activity.

Variations on the SCF theme

Despite the allure of the F-box hypothesis, individual components of the SCF system are also used in other configurations, not all of which are implicated in ubiquitin-dependent proteolysis (Table 1).

The CBF3 complex

Skp1p plays an essential role in the CBF3 kinetochore complex that binds the CDEIII element of yeast centromeres[14,15]. Skp1p was isolated from purified CBF3 complexes, which also contain Ctf13p, Cep3p and Ndc10p (Ref. 15), and by high-copy suppression of the *ctf13-30* mutation[14]. Reconstitution of active CBF3 requires Skp1p, which binds to the F-box of Ctf13p (Ref. 45). However, the Skp1p–Ctf13p interaction radically departs from the standard SCF configuration in that Skp1p is apparently not an obligate structural component of active CBF3. Rather, Skp1p appears to facilitate an essential modification of Ctf13p by an as yet unidentified kinase[45]. In addition, Cdc34p and Cdc53p are not evident in purified CBF3 complexes[15,45]. Three possible but not mutually exclusive explanations for Skp1p function in the CBF3 complex come to mind: Skp1p might act in a chaperonin-like fashion to fold Ctf13p into an active conformation that allows its phosphorylation; Skp1p might bridge Ctf13p to its activating kinase; or, Skp1p might actually target Ctf13p itself for degradation. The last possibility might tie in with the observations that overproduction of Cdc34p suppresses the *ndc10-1* mutation[46], and that Ctf13p is rapidly degraded in a Cdc4p- and Cdc34p-dependent manner[45]. Ctf13p is the only unstable component of CBF3 and this instability might provide a mechanism whereby active CBF3 is titrated against kinetochore number[45].

Elongin and VHL complexes

A tripartite complex of elongin A, elongin B and elongin C from human cells stimulates transcriptional elongation *in vitro*[47]. Intriguingly, elongin A contains an F-box, elongin B contains a ubiquitin-like domain, and elongin C is a Skp1p homolog[12]. The elongin B–C subcomplex also forms a separate complex with a Cdc53p homolog called CUL2 and the von Hippel Lindau (VHL) tumor suppressor gene product[48]. VHL is required for appropriate cell-cycle exit upon serum withdrawal and might serve to destabilize hypoxia-induced mRNAs, which are expressed inappropriately in $VHL^{-/-}$ cancer cells[49,50]. Elongin B–C appears to bridge CUL2 to VHL in a manner loosely analogous to the SCF configuration[50]. However, VHL does not contain an obvious F-box, and moreover, the elongin C binding site in elongin A might not overlap its F-box[47]. The relationship between the two different elongin B–C complexes is unclear, and as yet there is no evidence that either complex functions as an E3.

The anaphase-promoting complex

The other E3 complex to emerge from cell-cycle studies is the APC, a cell-cycle regulated E3 that is active from anaphase until the end of G1 phase (Fig. 1). The APC targets proteins containing a short primary sequence determinant called the destruction box[6]. The APC is a conserved multiprotein complex minimally composed of eight stoichiometric subunits in *Xenopus* and 12 subunits in yeast[51,52]. Genetic analysis in several organisms suggests that, like SCF complexes, the APC appears to utilize subfamilies of WD40 repeat proteins as cofactors in the degradation of specific substrates. In budding yeast, Cdc20p is a rate-limiting factor for degradation of the anaphase inhibitor Pds1p (Ref. 53), while slightly later in mitosis, a related WD40 repeat protein called Hct1p (Cdh1p) activates degradation of B-type cyclins and a spindle-associated protein called Ase1p (Refs 53, 54). Homologs of Cdc20p and Hct1p (Cdh1p) appear to play similar roles in other organisms[55]. Intriguingly, the Cdc20p family is at a nexus of the mitotic degradation and checkpoint pathways, as its activity is inhibited through physical interaction with the mitotic checkpoint protein Mad2p (Refs 56, 57). Whether F-box proteins might be subject to similar regulatory mechanisms remains to be investigated.

A second parallel between the APC and SCF complexes arose unexpectedly with the recent finding that the Apc2p subunit of the yeast APC complex is a Cdc53p homolog[51,52,58]. Conditional *apc2* mutations cause arrest in metaphase because of a failure to degrade Pds1p, as in other bona fide APC mutants[51,52,58]. Apc2p is only distantly related to Cdc53p, with most of the similarity restricted to a 200 amino acid internal region that encompasses the Cdc34p binding site in Cdc53p (Refs 20, 51, 52). It is tempting to speculate that this region in Apc2p binds the cognate E2 enzyme for the APC. Neither an F-box protein, nor a Skp1p homolog have emerged from exhaustive analysis of the APC, but by analogy to Cdc53p, Apc2p might yet function as a scaffold protein for the presumed substrate recruitment factors Cdc20p and Hct1p (Cdh1p).

SCF pathways in development

Recent genetic analysis in several model systems has revealed intriguing developmental roles for potential SCF complexes.

Plants

In *Antirrhinum majus* (snapdragon) an F-box protein encoded by *fimbriata* (*fim*) regulates the expression of *deficiens* (*def*), a member of the MADS-box family of transcription factors that is required for the development of petals and stamens[59]. FIM binds three Skp1p homologs (FIM-associated proteins, FAP1, -2, -3)[59], and if the F-box model holds, FIM might target a transcriptional repressor of *def* (and other homeotic genes) for proteolysis. Interestingly, expression of *fap1, -2, -3* is developmentally controlled in a pattern that only partially overlaps with *fim*, suggesting that expression patterns might regulate the composition of these putative SCF complexes[59]. In *Arabidopsis thaliana* an analogous developmental pathway is based on the *fim* homolog *UNUSUAL FLORAL ORGANS* (*UFO*)[60]. Another potential SCF complex in *Arabidopsis* has recently been implicated in hormonal signaling as an LRR containing F-box protein called TIR1 appears to mediate responses to the plant hormone auxin[61].

Caenorhabditis elegans

Genetic analysis in *C. elegans* has yielded two tantalizing insights into possible developmental roles for SCF complexes in animal cells. The first Cdc53p homolog characterized in metazoans is encoded by *cul-1*, the namesake of mammalian cullins[21]. *cul-1* was identified from a lineage mutant (formerly *lin-19*) that exhibited the unusual property of hyperplasia in all tissues of the normally invariant cell lineages of *C. elegans*[21]. By analogy to SCF^{Grr1}, CUL-1 might restrain division by targeting

G1 cyclins for ubiquitination, but this model remains speculative.

The first *C. elegans* F-box protein assigned a function is encoded by *sel-10*, which was identified in a screen for suppressors of partial loss-of-function alleles of the Notch receptor family member, *lin-12* (Ref. 62). One function of *lin-12* is to dictate a lateral fate decision between two cells of the hermaphrodite gonad in which one becomes the vulval anchor cell (AC) and the other a ventral uterine cell (VU). In the absence of sufficient *lin-12* activity, the default decision is for both cells to adopt the AC fate, a phenotype that is suppressed when *sel-10* activity is compromised. SEL-10 has similar overall structure to Cdc4p and physically interacts with the intracellular domain of LIN-12 and other Notch homologs, consistent with a targeting role for SEL-10 in LIN-12 proteolysis[62].

Drosophila melanogaster

Another Cdc4p homolog, Slimb, was isolated in a screen for recessive mutations that alter adult patterning in *Drosophila*. Loss of Slimb activity causes supernumerary limbs, which is indicative of ectopic Hedgehog (HH) and Wingless (WG) activity[63]. *slmb* mutants accumulate high levels of the downstream transcription factors in the HH and WG pathways, Cubitus interruptus (CI) and Armadillo (ARM), respectively[63]. Thus, the HH and WG pathways may regulate gene expression through inhibition of Slimb-mediated proteolysis. Although it is not known if proteolysis of CI and ARM is ubiquitin-dependent, the mammalian homolog of ARM, β-catenin, is degraded by the ubiquitin-proteasome pathway[64].

Viruses

As is often their wont, viruses can subvert cellular ubiquitination pathways to their own nefarious developmental ends, as for instance when the E3 ligase E6AP is commandeered by the HPV E6 protein to eliminate the TP53 (p53) tumor suppressor[2]. In the one spectacular SCF example so far, the Vpu protein of HIV downregulates CD4 expression in T-helper cells by targeting CD4 for ubiquitin-dependent proteolysis. Vpu binds CD4 and recruits it to an F-box protein called βTRCP (Ref. 65). The Vpu–βTRCP

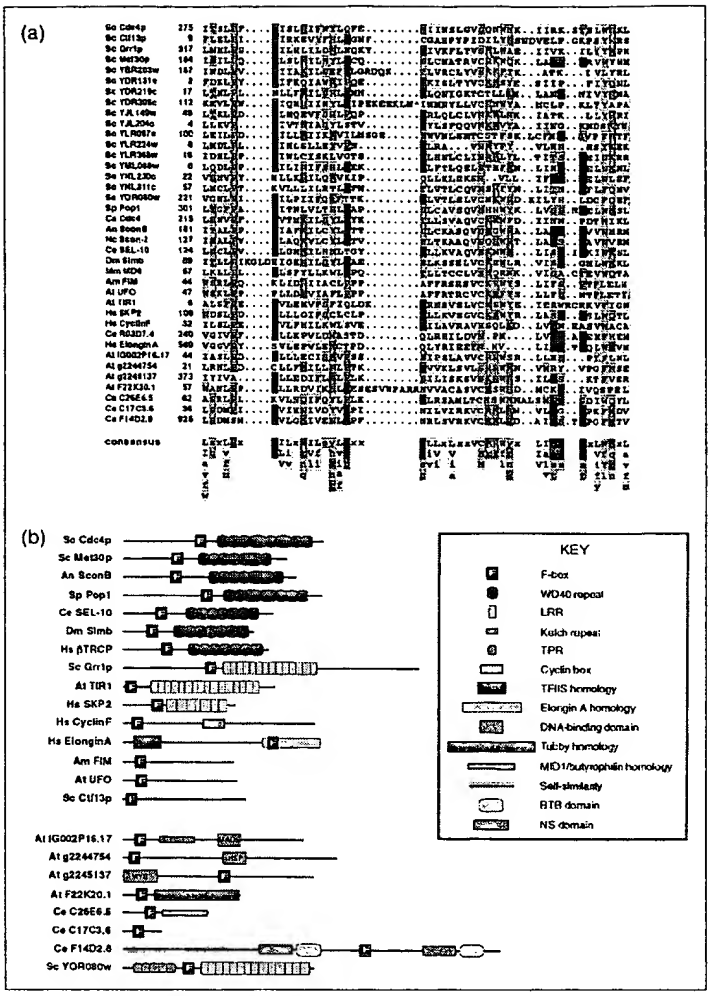

FIGURE 3. F-box proteins. (a) F-box alignment. The upper portion of the alignment shows all F-boxes identified in the *Saccharomyces cerevisiae* genome. The lower portion contains a sample of interesting F-box proteins, which are represented in (b), or are homologs of characterized F-box proteins in other organisms. Sequences in parts (a) and (b) were obtained from PSI BLAST searches performed at the NCBI (Ref. 69), and were analyzed by ClustalX (Ref. 70) followed by manual re-adjustment. The F-box motif shown here is extended by three N-terminal residues compared with the F-box alignment of Bai *et al.*[12] Note that most of the aligned F-box proteins have not yet been demonstrated to bind Skp1p or Skp1p-related proteins. An insertion of 11 residues in YDR306c is indicated (^). Organisms: Am, *Antirrhinum majus*; An, *Aspergillus nidulans*; At, *Arabidopsis thaliana*; Ca, *Candida albicans*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*. (b) Schematic representation of various F-box proteins. The proteins in the upper group have been characterized and are described further in Table 1. Uncharacterized F-box proteins that were identified in PSI BLAST searches[69] and in which other domains of interest were also identified are represented in the bottom group. The following domains or putative domains were identified in F-box proteins: WD40 repeats[16]; LRRs (Ref. 17); Kelch repeats[67]; TPRs (Ref. 66); TFIIS similarity[47]; tubby homology[71]; MID1 (butyrophilin) homology[72]; BTB domains[68]; and NS domains[73]. Note that we also found the NS domain in a subclass of human and yeast ubiquitin-specific proteases, such as HAUSP (herpes virus-associated ubiquitin-specific protease), which associates with a herpes virus RING-finger protein[74]. The elongin A homology region represents the region conserved between Hs Elongin A, Ce R03D7.4 and Sc YNL230c, and includes the F-box in all three proteins. Abbreviations: LRR, leucine-rich repeat; TPR, tetratricopeptide repeat; BTB, bric à brac/tramtrack/Broad-Complex; NS, N-terminus of SPOP.

interaction requires phosphorylation of Vpu, which drives its association with the WD40 repeats of βTRCP. Skp1p interacts with human βTRCP in an F-box-dependent manner, and this interaction is necessary for Vpu-induced degradation of CD4 (Ref. 65). As pointed out by Bai *et al.*[12], it is probably not insignificant that other F-box proteins and Skp1p homologs are encoded in viral genomes.

Substrate recruitment domains in F-box proteins

Specificity in F-box protein function probably derives in large part from the protein–protein interaction domains that recruit various substrates for ubiquitination. WD40 repeats[16] and LRRs[17] still top the list in F-box proteins of known function. However, the repertoire of F-box proteins has expanded considerably since the original compilation of F-box proteins[12], and in parallel new potential protein–protein interaction domains have emerged. In PSI BLAST searches seeded with subsets of F-box proteins in current databases, we could identify at least seven additional domains that had been previously unnoticed in F-box proteins (Fig. 3b). Of these, the best characterized are TPR repeats[66], which mediate protein–protein interactions in several systems, including the APC, and kelch repeats[67], which often occur in actin-interacting proteins. Intriguingly, several F-box proteins contain domains that are implicated in transcriptional regulation including various DNA-binding domains, the BTB domain[68], and the TFIIS/elongin A homology region[47]. Many of the WD40 and LRR containing F-box proteins described above are also directly or indirectly implicated in transcriptional control. While the precise functions of many of the domains shown in Fig. 3 remain to be determined, the F-box hypothesis predicts that such domains will mediate protein–protein interactions.

The SCF numbers game

How far can the F-box hypothesis be pushed? It is likely that other SCF configurations will occur in yeast, because at least 13 other yeast proteins contain a recognizable F-box motif (Fig. 3a). Indeed, we have found that two F-box proteins of unknown function form complexes with Cdc53p in yeast lysates (A.R. Willems, A. Shevchenko and M. Tyers, unpublished). In addition to Cdc53p and Apc2p, yeast also contains two other Cdc53p homologs of unassigned function, as well as an elongin C homolog. The combinatorial possibilities in metazoans are perhaps best illustrated in *C. elegans* for which PSI BLAST database searches reveal seven Cdc53p homologs, 10 Skp1p homologs, and at least 60 F-box-containing proteins!

Key issues to resolve at this point include: identification of the putative substrates for known SCF complexes; assignment of function to F-box proteins revealed by the genome sequencing projects; and the role of the Skp1p–F-box interaction in non-SCF contexts. There is also no shortage of substrates begging for an SCF complex. Of these, the phosphorylation-dependent pathways that target mammalian G1 cyclins, CDK inhibitors and cell-cycle transcription factors are prime examples. Given that the human SCF^{Skp2} complex bears many similarities to SCF complexes in yeast[35], expectations are high that the mammalian pathways will soon fall into place[22]. Whatever the precise rules that govern the permutations and combinations, F-box proteins and their cohorts are a sure bet for many interesting stories to come.

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In accord with *TIG* editorial policy, individual yeast proteins are designated by the suffix 'p', consistent with the protein nomenclature convention recommended by the Committee for Genetic Nomenclature for *S. cerevisiae*. Families of yeast proteins are designated in uppercase letters. The authors wish to note, however, that they do not endorse the 'p' nomenclature convention.

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SKP1 Connects Cell Cycle Regulators to the Ubiquitin Proteolysis Machinery through a Novel Motif, the F-Box

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Summary

We have identified the yeast and human homologs of the *SKP1* gene as a suppressor of *cdc4* mutants and as a cyclin F–binding protein. Skp1p indirectly binds cyclin A/Cdk2 through Skp2p, and directly binds Skp2p, cyclin F, and Cdc4p through a novel structural motif called the F-box. *SKP1* is required for ubiquitin-mediated proteolysis of Cln2p, Clb5p, and the Cdk inhibitor Sic1p, and provides a link between these molecules and the proteolysis machinery. A large number of proteins contain the F-box motif and are thereby implicated in the ubiquitin pathway. Different *skp1* mutants arrest cells in either G1 or G2, suggesting a connection between regulation of proteolysis in different stages of the cycle.

Introduction

Cell cycle transitions are regulated by the activation and inactivation of cyclin-dependent kinases (Cdks). Cdks are regulated by association with positive regulatory subunits known as cyclins, negative regulators known as Cdk inhibitors (CKIs) and by phosphorylation (reviewed by Harper and Elledge, 1996). The balance of these factors control Cdk activity and serve to integrate signals intended to coordinate cell cycle transitions. The levels of these proteins are tightly regulated both transcriptionally and post-translationally, the latter primarily achieved by regulated ubiquitin-mediated proteolysis (Glotzer et al., 1991; Schwob et al., 1994; Pagano et al., 1995; reviewed by Deshaies, 1995). The formation of ubiquitin–protein conjugates usually requires the sequential action of three enzymes. A ubiquitin-activating enzyme, E1, uses ATP to form a thioester bond between itself and ubiquitin. E1 then transfers ubiquitin to a ubiquitin-conjugating enzyme, E2. Finally, ubiquitin can be transferred to the substrate either directly by an E2 or by an E2 acting together with a ubiquitin–protein ligase, E3. In the latter case, it is not clear whether the E2 transfers the ubiquitin to the E3, or whether the E3 acts as a specificity factor to recruit substrates for the E2. The end result is the polyubiquitination of proteins, which targets them for destruction by a complex protease called the proteosome. Protein degradation is a particularly effective method for promoting unidirectional progression in the cell cycle because of its irreversibility. Currently, three major cell cycle transitions, entry into S phase, separation of sister chromatids, and exit from mitosis, are known to require the degradation of specific proteins.

Sic1p inhibits Clb/Cdc28 kinases and its destruction is required for S phase entry (Schwob et al., 1994). Sic1p is synthesized late in mitosis and is destroyed after START (Donovan et al., 1994). In addition to active Cln/Cdc28, Sic1p destruction requires *CDC4*, *CDC53*, and *CDC34*. Mutants in these three genes arrest with unreplicated DNA and multiple buds and show a common pattern of suppression and enhancement indicating a role in a common process (Mathias et al., submitted). Deletion of *SIC1* allows DNA replication to proceed in these mutants, but does not suppress lethality (Schwob et al., 1994). *CDC34* encodes an E2 ubiquitin-conjugating enzyme (Goebl et al., 1988) that can catalyze the formation of polyubiquitin on itself and other substrates (Banerjee et al., 1993; Deshaies et al., 1995). *CDC34* has also been implicated in Cln2p destruction (Tyers et al., 1992; Deshaies et al., 1995). *CDC4* encodes a protein with WD40 repeats (Yochem and Byers, 1987).

Mitotic cyclin proteolysis is also essential for cell cycle progression. A- and B-type cyclins contain a motif known as a destruction box that confers regulated ubiquitin-mediated destruction at distinct points in the cycle (Glotzer et al., 1991). Cells expressing mutants lacking the destruction box arrest in anaphase (Holloway et al., 1993; Surana et al., 1993). Cyclin F shows tightly regulated abundance like cyclin A, but lacks a canonical destruction box motif (Bai et al., 1994). Cells overproducing cyclin F accumulate in G2. In frog and clam egg extracts, mitotic cyclin destruction involves a large E3 complex referred to as the cyclosome or anaphase promoting complex (APC) (Hershko et al., 1994; King et al., 1995; Sudakin, et al., 1995). In yeast, *CDC16*, *CDC23*, *CDC27*, and *CSE1* are involved in mitotic cyclin destruction (Irniger et al., 1995) and homologs of *CDC16* and *CDC27* are found in the Xenopus APC. So far, components of the G1/S and G2/M proteolysis pathways appear to be distinct.

In addition to cyclins and CKIs, other cell cycle–regulated targets for destruction include proteins that control sister chromatid separation at kinetochores (Holloway et al., 1993; Irniger et al., 1995; King et al., 1995; Tugendreich et al., 1995). Ubiquitin-mediated proteolysis is required for anaphase entry and, while cyclin B destruction normally occurs then, cyclin B destruction is not required for that transition. The separation of sister chromatids can proceed in the presence of mutant cyclins that cannot be degraded in mitosis (Holloway et al., 1993; Surana et al., 1993), indicating a second proteolysis target for sister chromatid separation. The identity of this target is unknown and it is an outstanding

question as to how the APC is targeted to kinetochores or cyclins.

How the timing and selectivity of destruction is controlled is also not clear. There is no evidence that *CDC34*-dependent ubiquitin conjugating activity is regulated, suggesting that the substrates themselves may be activated. Support for this hypothesis comes from the fact that *cln2* mutants lacking Cdk phosphorylation sites have greatly enhanced stability (Lanker et al., 1996). For mitotic cyclin destruction, it appears that the APC is activated at mitosis by mitotic cyclin/Cdks (Hershko, et al., 1994; King et al., 1995; Sudakin, et al., 1995) and inactivated at START by G1 cyclin/Cdks (Amon, et al., 1994). Moreover, subtle substrate-specific timing differences exist, e.g., cyclin A and cyclin B have a very similar destruction box sequence but cyclin A is degraded prior to cyclin B (Evans et al., 1983; Luca et al., 1991; Hunt et al., 1992). Destruction timing may involve differences in either localization of the cyclin/Cdk complexes or the proteins with which they associate. Cyclin/Cdk-specific binding proteins such as the CKIs show specificity for cyclin/Cdk binding (Harper and Elledge, 1996). Furthermore, two novel proteins, Skp1p and Skp2p, have been found associated with cyclin A/Cdk complexes but not cyclin B/Cdk complexes (Zhang et al., 1995). These proteins are candidates for mediators of stage- and cyclin-specific ubiquitin-dependent destruction.

Here, we report the identification of a yeast gene, *SKP1*, that is required for both the G1/S and G2/M transitions and is involved in the proteolysis of the Sic1p protein through interactions with Cdc4p. Skp1p has also been found to associate with cyclins A and F, and with kinetochores in yeast (Connelly and Hieter, 1996). Thus, *SKP1* may provide a common link between these molecules and the proteolysis machinery.

Results

A Yeast and Human Gene Linked to Cell Cycle Regulators: Connections between Suppressors of *cdc4* Mutants

Human cyclin F was originally identified as a suppressor of a *cdc4-1* mutant (Bai et al., 1994). Two independent

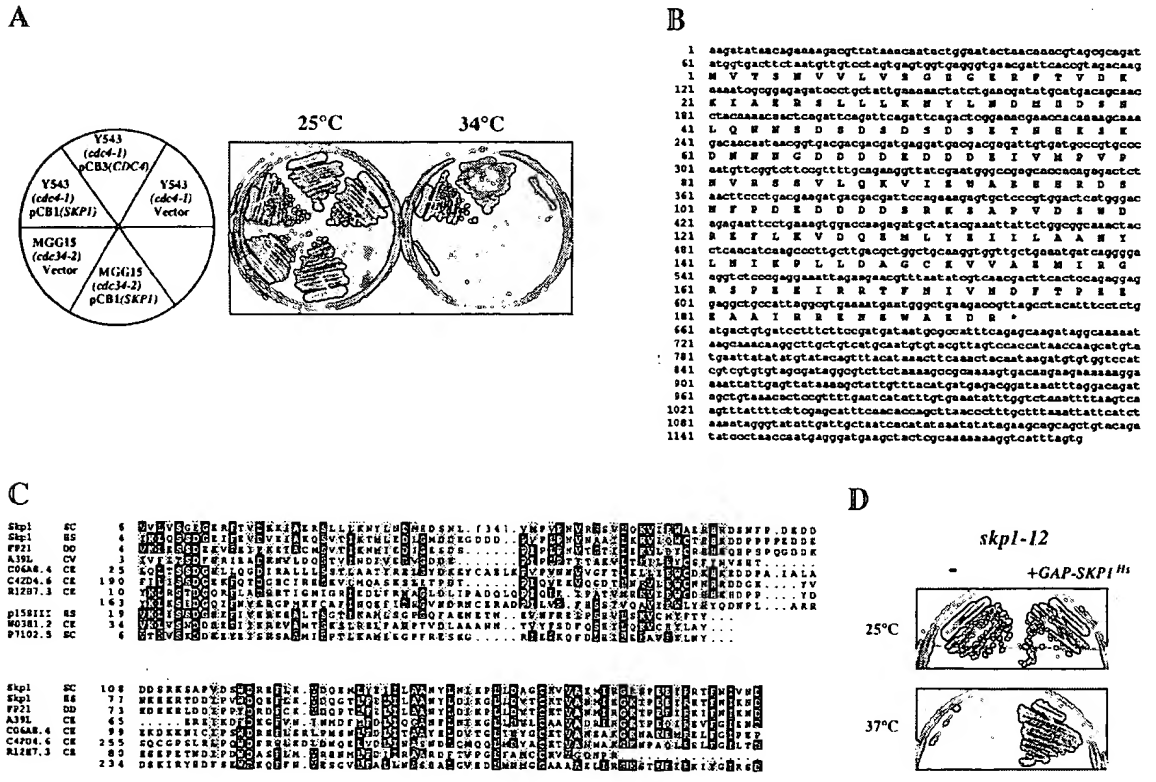

Figure 1. Isolation of the Yeast and Human *SKP1* Genes

(A) *SKP1* overproduction suppresses the temperature sensitivity of *cdc4-1* mutants. Y543 (*cdc4-1*) cells containing vector alone, pCB1 (*GAL–SKP1*), pCB2 (*GAL–CDC4*), or pCB3 (*GAL–CLB4*), or YL10 (*cdc34-2*) cells with pCB1 or vector alone were struck on SC–uracil plates containing galactose as a carbon source and incubated at 25°C or 34°C for 4 days. pCB1 (*GAL–SKP1*) failed to suppress *cdc4-1* when grown on media containing glucose as a carbon source (data not shown).

(B) DNA and deduced protein sequence of *SKP1*.

(C) Alignment of *SKP1* homologs and related genes. Amino acid identities are shown as black boxes. Conservative changes are shown as shaded boxes. S. cerevisiae *SKP1* has a 34 amino acid insertion of primarily N, D, and E amino acids in the N-terminal domain. DD is Dictyostellium discoidium, SC is S. cerevisiae, HS is Homo sapiens, CV is Chlorella Virus, and CE is C. elegans.

(D) Human Skp1p can complement the temperature sensitivity of a *skp1-12* mutant. Human *SKP1* under *GAP* promoter control on pAB23 or pAB23 alone in Y555 (*skp1-12*) (see below) was streaked onto SC–uracil plates and incubated at 25°C or 37°C as indicated.

lines of research, one on cyclin F and one on *CDC4* identified the human and yeast homolog of the same gene, *SKP1*. In a search for yeast overproduction suppressors of *cdc4-1* using a Saccharomyces cerevisiae cDNA library driven by the *GAL1* promoter (Liu et al., 1992), two genes were identified: *CLB4* (Bai et al., 1994) and a novel gene, *SKP1* (Figures 1A and 1B).

We also identified the human homolog of *SKP1* in a search for cyclin F binding proteins using the two-hybrid system (Durfee et al., 1993; Harper et al., 1993). During the course of this work, human SKP1 was independently identified as a cyclin A/Cdk2-associated protein called S phase kinase-associated protein (Skp1p) (Zhang et al., 1995). Skp1p binds to Skp2p, which binds directly to cyclin A/Cdk2 complexes. While Skp2p is essential for S phase entry, the function of Skp1p was unknown. Yeast and human *SKP1*, which share 48% identity, are members of a large family of proteins that have two domains connected by a linker region (Figure 1C). The N-terminal domain is related to RNA polymerase elongation factor p15SIII; the second domain is specific to *SKP1* proteins. Homologs of *SKP1* are found in many species (Figure 1C), and three homologs exist in Caenorhabditis elegans; one (R12H7.3) has a direct repeat of both domains of Skp1p. Yeast Skp1p has a 30 amino acid insertion in the N-terminal domain relative to human Skp1p. This difference is not critical because high level expression of the human gene can complement the temperature sensitivity of *skp1-12* mutants (Figure 1D).

Yeast *SKP1*, a Suppressor of a Cdk Inhibitor Proteolysis Defect

cdc4-1 mutants arrest at the restrictive temperature with high levels of Sic1p that inhibit the Clb5 and Clb6/Cdc28 complexes required for S phase entry. Deletion of *SIC1* allows *cdc4* mutants to enter S phase (Schwob et al., 1994). Given these results, *CLB4* and cyclin F suppression of *cdc4* mutants is likely due to their potential for producing active B-type cyclin/Cdk complexes that titrate out Sic1p. Since human Skp1p can potentially associate with cyclin F, it raised the possibility that *SKP1* was working in a similar manner to suppress *cdc4*. If these *cdc4* suppressors are bypassing Sic1p destruction, they should have high levels of Sic1p. Sic1p levels in *cdc4-1* mutants were measured in the absence or presence of plasmids expressing *CDC4*, *CLB4*, *SKP1*, and cyclin F. *cdc4* mutants expressing *CLB4* or cyclin F retain high Sic1p levels equivalent to the vector alone control, consistent with bypass suppression. *CDC4* or *SKP1* expressing cells showed low Sic1p levels (Figure 2). This indicated that *SKP1* was suppressing *cdc4* mutants by a different mechanism, most likely by reactivation of the *cdc4*-dependent Sic1p destruction pathway.

SKP1 and *CDC4* Show Specific and Reciprocal Suppression Patterns

Although *CDC4*, *CDC34*, and *CDC53* function in the same pathway, *SKP1* overproduction does not suppress *cdc34-2* (see Figure 1A) or *cdc53-2* mutants (data not shown), indicating specificity for *CDC4*. Furthermore, in a screen to identify cDNAs capable of suppressing a Ts *skp1* mutant, we found that *CDC4* overexpression could suppress *skp1-11* but not *skp1-12* mutants (data not shown). Thus, *SKP1* and *CDC4* are capable of reciprocal suppression.

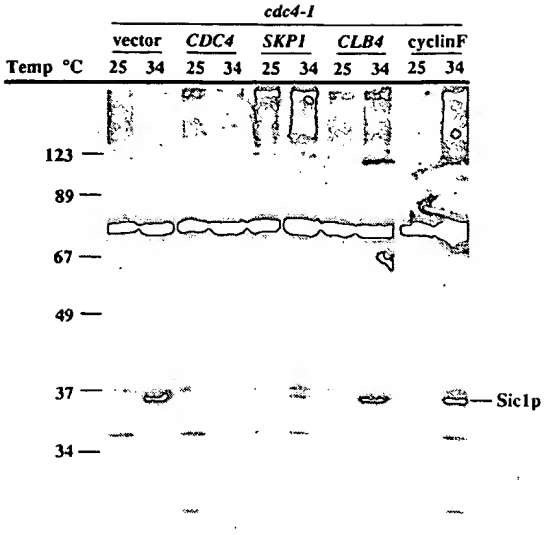

Figure 2. *SKP1* Overproduction Restores the Ability of *cdc4-1* Mutants to Destroy Sic1p

Y543 (*cdc4-1*) cells containing pCB1 (*GAL–SKP1*), pCB2 (*GAL–CDC4*), pCB3 (*GAL–CLB4*), or pCB30 (*GAP*–cyclin F) were grown to log phase at 25°C in SC–uracil media containing galactose as a carbon source and shifted to nonpermissive temperature, 34°C. Total protein was isolated 5 hr after the temperature shift. 100 μg of protein was loaded on each lane (SDS–PAGE), transferred to nitrocellulose, and immunoblotted with anti-Sic1p antibodies (Donovan et al., 1994). Protein was detected using enhanced chemiluminescence.

Cyclin F, Cdc4p, and Skp2p Share a Structural Motif, the F-Box, that Is Responsible for Binding to Human and Yeast Skp1p

Sequence analysis of cyclin F revealed a motif we have named the F-box that is shared by Cdc4p and Skp2p and which may represent a Skp1p interaction domain. To examine whether Cdc4p binds Skp1p in vivo, yeast cells expressing a biologically active glutathione S-transferase (Gst)–Skp1p were made. Cdc4p is found in Gst–Skp1p affinity purified complexes (Figure 3A). To test whether this association was direct, in vitro translated Cdc4p was incubated with bacterially expressed Gst–Skp1p bound to glutathione beads. Strong retention of Cdc4p was observed with Gst–Skp1p, while only trace levels were bound by Gst alone (Figure 4C).

The ability of Skp1p to bind cyclin A/Cdk2 and cyclin F was also examined. As shown previously (Zhang et al., 1995), Skp1p association with cyclin A/Cdk2 was dependent upon the presence of Skp2p (see Figure 3B). In contrast, large amounts of Skp1p bound to Gst–cyclin F in the absence of Skp2p (see Figure 3B). Coomassie staining of bound proteins revealed that cyclin F and Skp1p were the most abundant proteins, suggesting a direct association (data not shown). While Skp2p can associate with cyclin F, it is not required for association of Skp1p with cyclin F. Thus, Skp1p can associate with multiple cyclins both directly and indirectly. We have

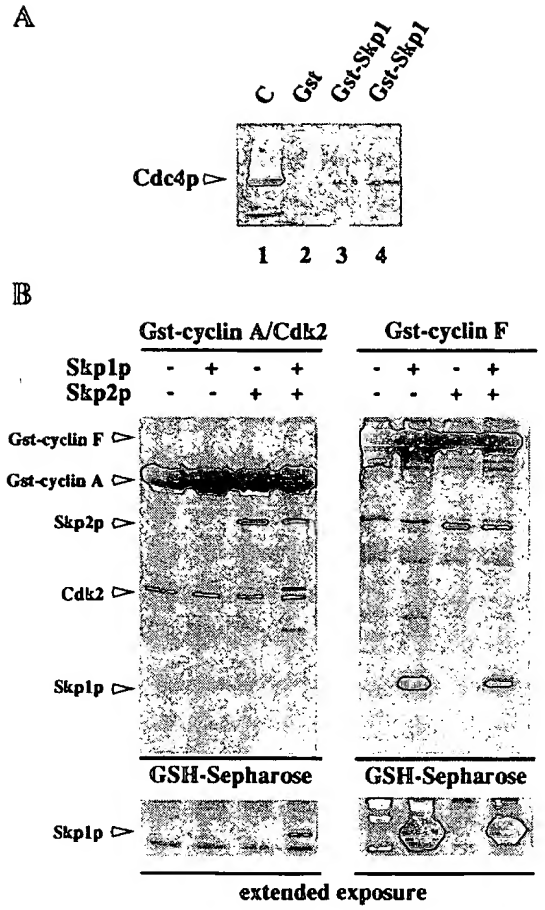

Figure 3. Association of Skp1p with Cyclin F and Cdc4p

(A) Protein was isolated from exponentially growing Y80 (wild type, lanes 1–3) carrying pADH–Gst vector alone or pCB23 (ADH–Gst–*SKP1*), or from Y549 (*skp1*Δ + pCB23) (lane 4) in SC–leucine media. Total yeast extracts were run as a blotting control in lane 1. In lanes 2–4, equal amounts of extracts were incubated with GSH–Sepharose and bound proteins immunoblotted with anti-Cdc4p antibodies. Coomassie staining revealed that two-fold more Gst than Gst–Skp1p was isolated.

(B) Association of cyclin F and Skp1p. Gst-cyclins and associated proteins were isolated from ^{35}S-methionine-labeled insect cells using GSH–Sepharose after co-infection with the indicated baculoviruses and separated by SDS–PAGE. Proteins were visualized by autoradiography after 10 hr (top) and 40 hr (bottom). The identity of Skp1p and Skp2p in these complexes was verified by immunoblotting with anti-Skp1p and anti-Skp2p antibodies (data not shown).

confirmed these results by showing that in vitro translated cyclin F can bind to bacterially produced Gst–Skp1p. In reciprocal experiments, we have shown that the bacterially expressed yeast Gst–Skp1p can bind to in vitro translated cyclin F and bacterially expressed human Gst–Skp1p can bind to Cdc4p (data not shown), underscoring the functional conservation between the Skp1p homologs.

The finding that all three proteins containing the F-box directly bound to Skp1p suggested the F-box was responsible for binding. Using this homology region, we searched all known databases and found 28 proteins containing this motif, nine from S. cerevisiae (Figure 4A). The similarity searches for the F-box motif definitions were done using generalized profiles (Bucher et al., 1996). Site-directed mutants in conserved residues were constructed to test whether the F-box is required for binding. Five mutants in the Cdc4p F-box were made: P279A, I286A, L290A, W316A, and a double mutant LP278-279AA. These mutant proteins were tested for binding to yeast Gst–Skp1p. Each mutation significantly decreased or abolished binding, indicating that the F-box is required for Skp1p association (Figure 4C). Data for the last two mutants are not shown but each had a significant decrease in binding. That this region is important for *CDC4* function is illustrated by the fact that the temperature-sensitive *cdc4-5* mutant changes an amino acid within the F-box, G300D, adjacent to a highly conserved F-box residue (J. Rosamond, personal communication).

SKP1 Is an Essential Gene Required for Both the G1-S and G2-M Transitions

To determine the genetic function of *SKP1*, a *skp1* deletion was made by precisely replacing the coding sequence with the *HIS3* gene, using one step gene replacement to make the diploid Y545. Sporulation and tetrad dissection showed 2:0 segregation for viability, and all viable spores were His$^-$, indicating that *SKP1* is an essential gene.

To further explore *SKP1* function, temperature-sensitive *skp1* mutants were made using hydroxylamine mutagenesis and a plasmid shuffling technique described in the Experimental Procedures. Two mutants unable to grow at 37°C were identified, *skp1-11* and *skp1-12* (Figure 5A). *skp1-11* mutants arrest with both unbudded and large budded cells with elongated buds (Figure 5C). Some of the large budded cells have multiple buds similar to *cdc4* mutants. Although the bud arrest morphology is not completely homogeneous, these cells are homogeneously arrested in G1 with a 1C DNA content (Figure 5B). This indicates that Skp1p is required for S phase entry.

Unlike *skp1-11* mutants, *skp1-12* mutants display a severe defect in progression through mitosis even at the permissive temperature, accumulating as large budded elongated cells with a G2 DNA content. After shift to the nonpermissive temperature, they initially maintain arrest as large budded cells with elongated buds. Their spindles are not completely homogeneous with a mixture of short and elongated spindles. After 3 hr of arrest, ~50% of the cells exit mitosis and arrest with a G1 content, indicating they also have a G1 defect. Incubation at 37°C for an additional 3 hr does not change the arrest profile of the DNA content, indicating that *skp1-12* mutants arrest in G1 and G2. These results indicate that *SKP1* is also required for proper mitosis.

skp1-11 carries two mutations (G539A and G560A), resulting in G160E and R167K changes in the C-terminal domain. Both residues are completely conserved among yeast, human, and C. elegans *SKP1* genes. *skp1-12* has a single base change (T83G) resulting in a leucine to glycine change (L8G) in the N-terminal domain. This

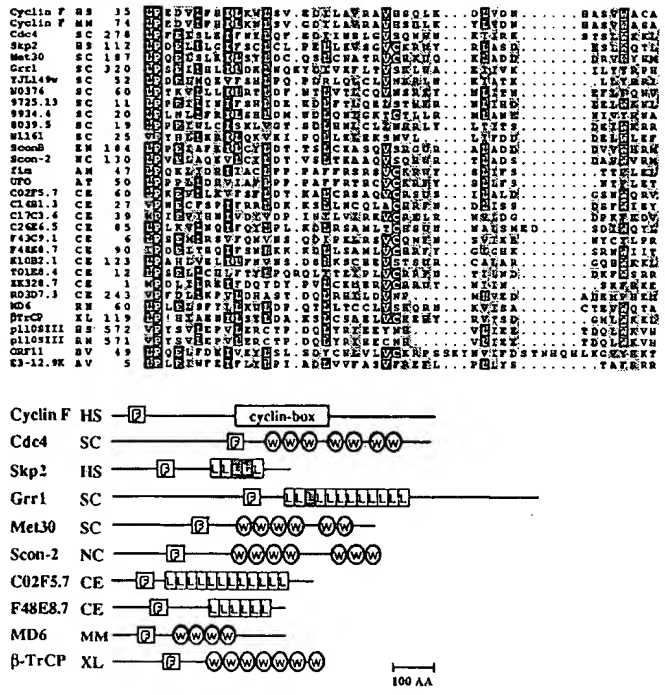

Figure 4. Binding of Skp1p Requires a Novel Structural Motif, the F-Box

(A) F-box alignment. A conserved region between Skp2p, Cdc4p, and cyclin F was identified and used to search the database for proteins containing this motif. Only sequences matching to established profiles with an error probability $p<0.03$ were accepted during iterative profile refinement and are shown here. Error probabilities were estimated as described in Hofmann and Bucher (1995).

(B) Schematic representation of additional motifs found in a subset of F-box proteins. F represents the F-box, W represents the WD40 repeat motif, and L represents the leucine-rich repeat motif.

(C) Mutants in conserved residues of the F-box fail to bind Skp1p. In vitro translated Cdc4p was used to bind with Gst–Skp1p. Site-specific mutations in conserved residues of the F-box, P279A, I286A, and L290A, were made as described in Experimental Procedures and labeled with ^{35}S-methionine by in vitro translation. These proteins were incubated with Gst or Gst–Skp1 protein and bound proteins were detected on SDS–PAGE by autoradiography. Lanes with Gst and Gst–Skp1p were incubated with 10 times the amount of in vitro labeled protein as compared with the in vitro translated lane.

suggests that the different domains of Skp1p may have distinct roles in different cell cycle phases. These mutants do not show interallelic complementation, indicating that they are defective in some common, as well as unique, functions (data not shown).

Sic1p Stability Is Increased in *skp1-11* Mutants

CDC4, *34*, and *53* have been implicated indirectly in Sic1p proteolysis. To examine whether Skp1p has a role in Sic1p destruction, the stability of Sic1p expressed from a plasmid was measured in wild-type and *skp1-11* mutants. Cells were arrested in S phase with hydroxyurea (HU) at a point where endogenous Sic1p transcription is repressed, shifted to 37°C, and *SIC1* expression was induced by addition of galactose. Transcription was repressed then by addition of glucose and Sic1p decay was measured over time by immunoblotting. A plasmid expressing *CLN2* from the *ADH* promoter was included because of the possibility that Sic1p phosphorylation by Cln/Cdc28 complexes is required for its destruction. Sic1p was unstable in Skp$^+$ cells with a half-life of 10 min or less and was significantly stabilized in *skp1-11* mutants (Figure 6A; data not shown). Both *skp1-11* and Skp$^+$ cells maintained the same DNA content throughout the experiment (Figure 6B), indicating that the differences in Sic1p stability are not due to differences in cell cycle position. Furthermore, endogenous Sic1p levels increase in *skp1-11* (but not *skp1-12*) mutants arrested at 37°C, although to a lesser extent than seen in *cdc4* mutants (data not shown). These results indicate that Skp1p is involved in Sic1p proteolysis.

skp1, *cdc4*, *cdc34*, and *cdc53* Mutants Arrest in G1 Because of Sic1p Accumulation and Are Sensitive to the Dosage of Sic1p

Since *skp1* mutants are defective in Sic1p proteolysis, overproduction of Sic1p might exacerbate the phenotype of *skp1* mutants. *SIC1* overproduced from *GAL1* promoter was lethal in *skp1-11* mutants at 30°C, but not in Skp$^+$ cells, consistent with a defect in Sic1p proteolysis (Figure 7A). Although Sic1p does not accumulate in *skp1-12* mutants, they may be weakly defective in its metabolism because *SIC1* overexpression causes slow

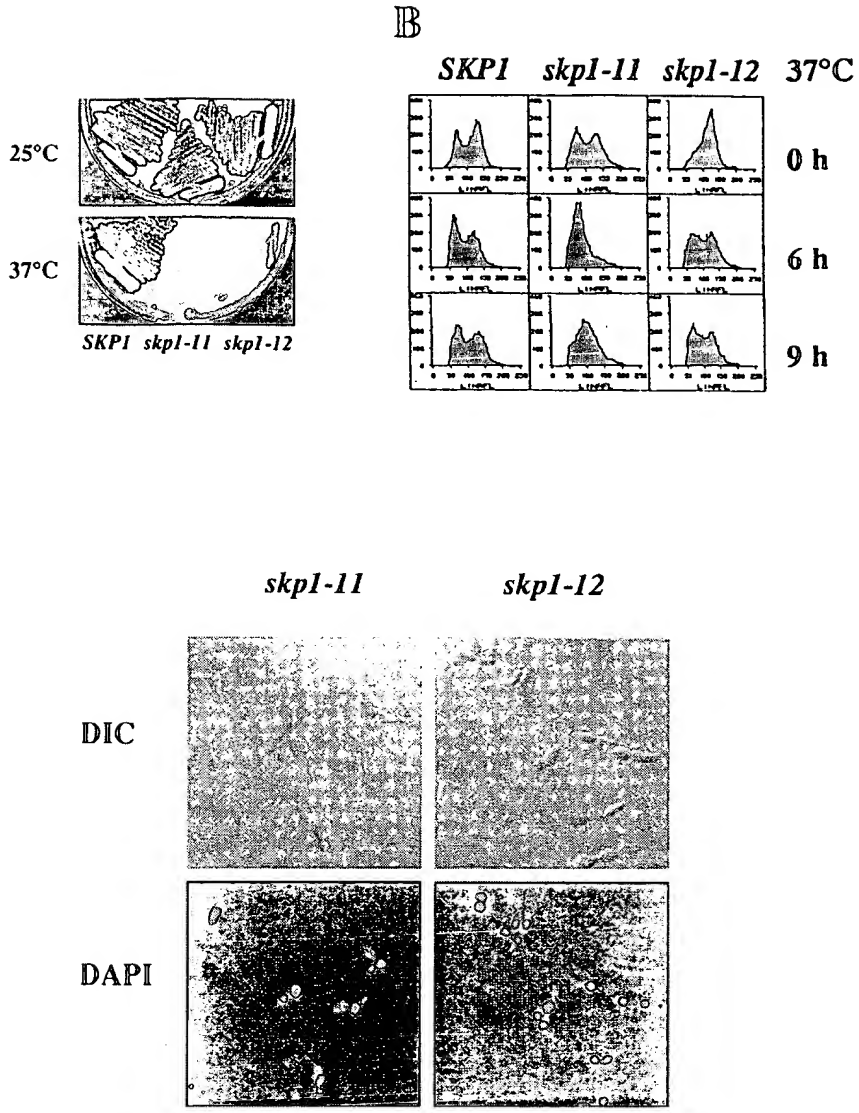

Figure 5. *skp1* Ts Mutations Are Defective in G1 and G2 Transitions

(A) Temperature-sensitive *skp i* mutants were generated using hydroxylamine mutagenesis and a plasmid shuffling protocol as described in Experimental Procedures. Y81 (*SKP1*), Y553 (*skp1-11*), and Y555 (*skp1-12*) mutant strains were struck onto YEDP plates and incubated at the permissive (25°C) or nonpermissive (37°C) temperatures and photographed after 2 days.

(B) Strains in (A) were incubated at 37°C for the indicated times and DNA content was determined by flow cytometry after propidium iodide staining.

(C) Phenotype of *skp1-11* and *skp1-12* mutants at the nonpermissive temperature. Photomicrographs of Y553 (*skp1-11*) and Y555 (*skp1-12*) cells grown in YEPD media at 25°C (OD_{600} = 0.1), shifted to 37°C, and incubated for 5 hr prior to fixation and staining. Nuclear morphology was visualized by DAPI.

growth at 34°C. *SIC1* overproduction is also lethal in *cdc4* and *cdc53* mutants and severely slows *cdc34* mutants, lending further support for a common defect in Sic1p proteolysis (Figure 7B). Examination of the phenotypes of *skp1-11* cells revealed that *SIC1* overproduction resulted in a small number of multiply budded cells in a wild-type background (5%) but a much higher percentage of multiply budded cells in a *skp1* mutant (28%). This is consistent with the interpretation that the *skp1-11* mutant is only partially defective for Sic1p proteolysis.

To test the hypothesis that *skp1-11* mutants are arrested prior to S phase owing to accumulation of Sic1p, we constructed *skp1-11 sic1::TRP1* double mutants. While *skp1-11* mutants exit an α-factor block but do not enter S phase, the *skp1sic1* double mutant enters S phase, albeit more slowly than wild type, and arrests in

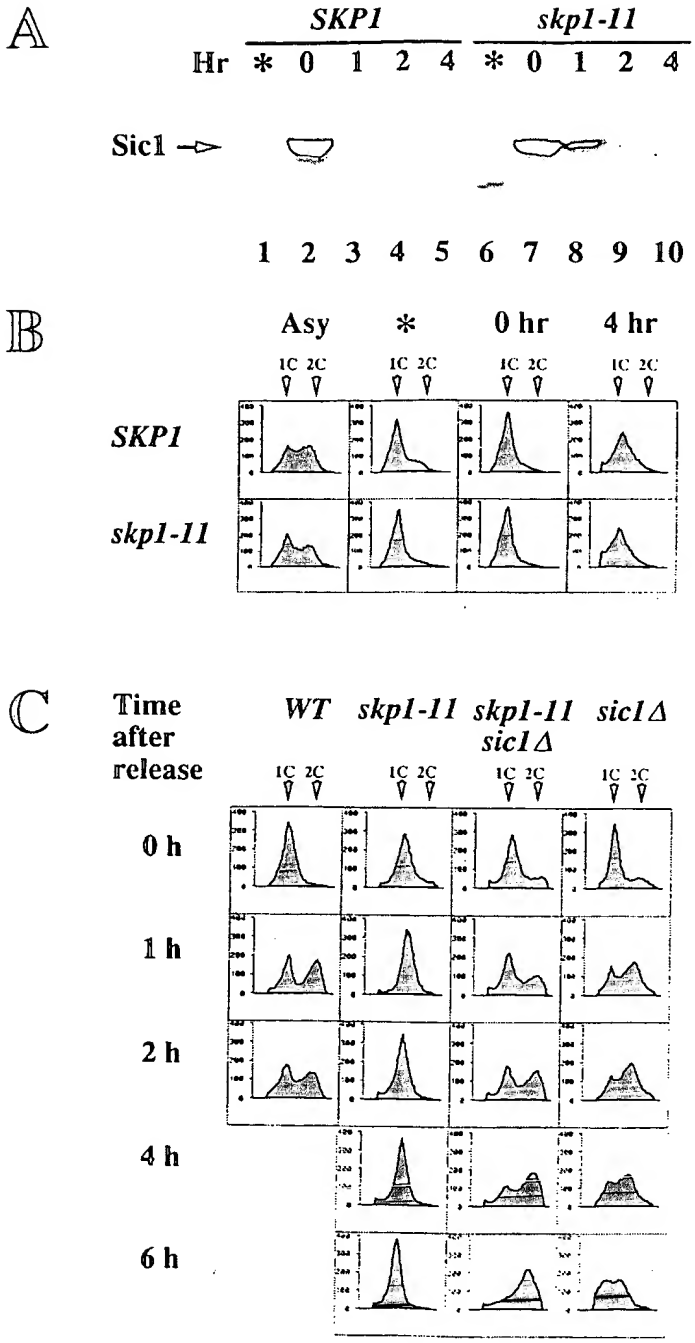

Figure 6. *SKP1* Controls Sic1p Stability

(A) Wild-type (Y81) and *skp1-11* (Y553) mutants containing pCB24 (*GAL–SIC1*) and pMT527 (*ADH–CLN2*) were grown in selective SC media containing raffinose and arrested in S phase with 200 mM HU for 2 hr. Galactose (2% w/v) was added for 1.5 hr to induce *SIC1* expression and cells were shifted to 37°C for 30 min. Cells were then resuspended in glucose media at 37°C (2% w/v) and samples were taken at the indicated time, protein prepared, and immunoblotted with anti-Sic1p antibodies. Lanes 1 and 6 marked by an asterisk above represent control samples arrested in HU just prior to addition of galactose.

(B) DNA content for the samples corresponding to the time points shown in (A). Asy refers to asynchronous samples prior to treatment with HU.

(C) Wild-type (Y80), *skp1-11* (Y552), *skp1-11 sic1Δ::TRP1* (Y563), and *sic1Δ::TRP1* (Y564) strains were grown at 25°C and synchronized in G1 with α-factor. Cells were shifted to 37°C for 90 min and then released from the α-factor block at 37°C. Samples were taken at the indicated time and DNA content determined.

G2 (see Figure 6C), indicating that Sic1p accumulation is responsible for the Cdc phenotype of *skp1-11* mutants.

skp1-12 Mutants Are Lethal in Combination with Cln2p Overproduction and Are Defective in Cln2p Proteolysis

Cyclins are among the main proteolysis targets important for cell cycle regulation. Therefore, we examined genetic interactions of *SKP1* with other potential proteolysis targets, the yeast cyclins. *CLN2* overexpression had a profound effect, killing *skp1-12* mutants at all temperatures (Figure 7A). *CLB2* overexpression was not toxic (data not shown). Since *CLN2* overproduction specifically kills *skp1-12* and not *skp1-11* mutants, it is likely due to an incompatibility with the defect unique to *skp1-12* mutants and may indicate that Cln2p metabolism is altered. *CLN2* overproduction is also toxic to *cdc34* mutants that are defective in Cln2p proteolysis (Des-

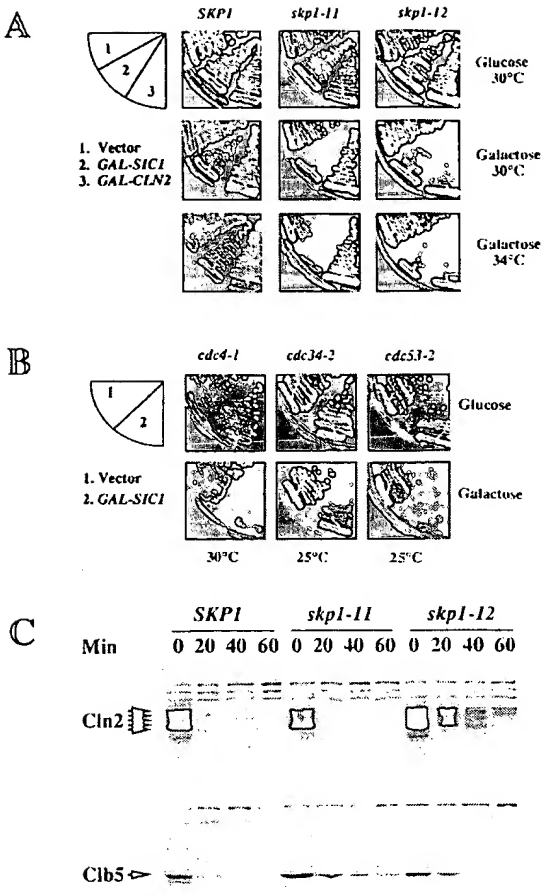

Figure 7. *SKP1* Controls Cyclin Stability

(A) Y81 (*SKP1*), Y553 (*skp1-11*), and Y555 (*skp1-12*) containing vector alone, or pCB24 (*GAL-SIC1*) or pMT634 (*GAL-CLN2*) were streaked upon SC−uracil media containing either glucose or galactose as a carbon source and incubated at the indicated temperature.

(B) Y543 (*cdc4-1*), YL10 (*cdc34-2*), and WX131-2c (*cdc53-2*) containing vector alone, or pCB24 (*GAL-SIC1*) were streaked upon SC−uracil media containing either glucose or galactose as a carbon source as indicated and incubated at the temperature indicated.

(C) Y556 (wild type), Y557 (*skp1-11*), and Y558 (*skp1-12*) containing pMT634 (*GAL-CLN2-3HA*) or pWS945 (*GAL-CLB5-3HA*) were grown in SC−uracil raffinose media and arrested in G1 with α-factor. Then cells were shifted to 37°C for 30 min before galactose was added (2% w/w final) and incubated for 1 hr to induce expression. Cells were resuspended in glucose media at 37°C (2% w/w) at time = 0 and samples were taken at 20 min intervals, protein prepared, and immunoblotted with anti-hemagglutinin antibodies.

haies et al., 1995). Alternatively, since Cln2p/Cdc28p activity is thought to inactivate APC in late G1, it is possible that the G2 defect in *skp1-12* is in APC function that is further exacerbated by *CLN2* overexpression.

To examine cyclin metabolism in these mutants, a Cln stability assay was performed at the nonpermissive temperature. Cells were arrested in G1, shifted to 37°C, and epitope-tagged cyclin expression was induced by addition of galactose. Cyclin transcription was then repressed by addition of glucose, and cyclin decay was measured over time by immunoblotting (Figure 7C). Both *skp1-11* and *skp-12* mutants show increased Clb5p stability. However, only *skp1-12* mutants showed a pronounced stabilization of Cln2p, consistent with the lethality observed by *CLN2* overproduction. Equivalent results were obtained for Cln2p destruction when cells were synchronized in S phase with HU (data not shown).

Discussion

Three cell cycle transitions require the degradation of specific proteins; entry into S phase (CKIs), separation of sister chromatids (anaphase inhibitory factors), and exit from mitosis (mitotic cyclins). Each of these requires degradation of one or more proteins essential for the transition and also other proteins whose presence may interfere with future events, e.g., Cln2p. In this study, we have identified an evolutionarily conserved gene, *SKP1*, that is connected to these three groups of functions and to the proteolysis machinery, possibly indicating a common mechanism for these events. The strongest evidence, summarized below, links yeast Skp1p to the proteolysis of the CKI Sic1p and the cyclins Cln2p and Clb5p. Additional data indicate its association with the other critical targets of cell cycle transitions. Mammalian Skp1p can bind to Skp2/cyclin A/Cdk2 and cyclin F. Both of these cyclins are unstable proteins. The significance of this binding has not been determined yet but is likely to provide a link to the proteolysis machinery. Skp1p is also associated with the kinetochore in yeast (Connelly and Hieter, 1996), the site of sister chromatid separation, another event dependent upon proteolysis. These connections indicate that *SKP1* is a molecule of central importance to a number of cellular processes.

The Role of *SKP1* in the G1 to S Phase Transition: Promoting CKI Degradation

Certain alleles of *skp1* specifically arrest in G1 and fail to properly degrade Sic1p although other START-dependent events, such as budding, occur. Failure to enter S phase is due to a defect in Sic1p degradation because deleting *SIC1* allows these cells to enter S phase and arrest with a G2 DNA content. One essential role in G1 is likely to involve facilitating the function of Cdc4p in promoting Sic1p proteolysis. This conclusion is supported by the following facts: *SKP1* overproduction suppresses a *cdc4-1* mutant; *CDC4* overproduction can suppress a *skp1-11* mutant; *skp1* mutants, like *cdc4* and *cdc53* mutants, are killed by overexpression of Sic1p; and Skp1p binds to Cdc4p in vitro and in vivo. Furthermore, Sic1p is the critical proteolysis substrate required for S phase entry in *skp1-11* mutants.

The Role for *SKP1* in the G2/M Transition: Promoting Proteolysis?

skp1-12 mutants are defective in mitotic entry. While we have no direct evidence that the function responsible for this defect involves proteolysis, this is a distinct possibility given the *skp1-12* defect in cyclin proteolysis,

the role of *SKP1* in G1/S, and the importance of proteolysis to mitosis. *SKP1* is involved in chromosome segregation and is present in kinetochore complexes (Connelly and Hieter, 1996). While speculative, it is possible that *SKP1* is involved in localizing the APC to kinetochores to promote destruction of proteins that maintain the cohesion of sister chromatids. Mutants defective in APC function such as *cdc16*, *cdc23*, and *cdc27* have chromosome segregation defects like *skp1* mutants. Alternatively, *SKP1* may facilitate ubiquitination that serves a regulatory function other than proteolysis.

Given its association with mitotic cyclins in mammals, *SKP1* may also play a role in metabolism of yeast mitotic cyclins. *SKP1* represents the first component of the G1/S proteolysis pathway that also functions in G2. It will be of interest to determine whether other members of the *CDC34/4/53* family also function in G2. The fact that a *cdc4sic1* double mutant arrests in G2 suggests that this whole complex may also be required for mitosis. However, this G2 arrest could result from the defective execution of a G1 function that triggers a G2 checkpoint.

The F-Box, a New Motif in Protein Degradation, Links Skp1p to a Large Number of Proteins

Skp1p binds to both human and yeast proteins through a conserved structural motif called the F-box. There are currently 28 genes in the database that contain many or all of the conserved residues in this motif that we have shown to be important for binding Skp1p. This is a conservative estimate since more divergent members may fail the stringent parameters of our analysis. This is a large number of genes and points to a potentially wide network of interactions. Since C. elegans has several *SKP1*-related genes, it is likely that mammals and other metazoans will also, adding further layers of complexity to the network.

Potentially interesting is the fact that 50% of F-box proteins have additional motifs involved in protein–protein interaction (Figure 4B), a frequency significantly higher than non-F-box-containing proteins. Four proteins, including Skp2p, have a series of leucine-rich repeats. Skp2p binds to both cyclin A/Cdk2 and to cyclin F, possibly through these repeats. One feature shared between the *SKP1*-associated cyclins A and F is the timing of their destruction. Both are destroyed prior to cyclin B (Evans et al., 1983; Bai et al., 1994). Cyclin A requires Cdk binding for destruction (Stewart et al., 1994) and for optimal Skp2p association. A second connection between *SKP* function and cyclin proteolysis comes from the observation that the gene most closely related to *SKP2* in yeast is *GRR1* (Flick and Johnston, 1991). Both proteins have the same leucine repeat motif and both contain an F-box (Figure 4B). *GRR1* is required for Cln1p and Cln2p proteolysis (Barral et al., 1995), suggesting that Skp2p might also function in an analogous manner with cyclin A. Whether these correlations are mere coincidences or indicate a link to the proteolysis machinery will require further experimentation. If a link to the proteolysis machinery exists, it could target cyclins or CKIs for destruction. Alternatively, Skp1p association could target active Cdks to targets in the proteolysis machinery. Cdk phosphorylation has been suggested to play a role in regulation of the APC (Lahav-Baratz et al., 1995).

Eight proteins, including Cdc4p and Met30p, have WD40 repeats. It is not known what Cdc4p binds through these repeats, although phosphorylated Sic1p is a possibility. Met30p associates with a transcriptional activator, Met4p, and negatively regulates its function in the presence of methionine (Thomas et al., 1995). It is not known whether this involves proteolysis of Met4p, although association with Skp1p raises this possibility.

Many F-box-containing proteins do not have additional motifs. Intriguingly, two viral proteins, adenovirus E3-12.9K and baculovirus ORF11, appear to essentially encode only an F-box. We also have observed that a *SKP1*-related gene is present on Chlorella virus (Figure 1C). Given the propensity of viruses to subvert cell cycle regulation to facilitate their replication, it is possible that the ubiquitin-mediated proteolysis pathway is also a target of viral manipulation. Perhaps by overproduction of an F-box, the virus can inhibit degradation of specific subsets of proteins, such as cyclins, to enhance its replicative capacity or promote the degradation of specific inhibitory proteins. Alternatively, these proteins may target the destruction of particular proteins antagonistic to virus survival.

Possible Roles of Skp1p in Cell Cycle–Regulated Destruction

We would like to propose a speculative model in which Skp1p may act as a central component of an E3 complex of proteins. In some circumstances, it may directly contact substrates, such as in the case of cyclin F, and regulate their destruction. Under other circumstances, it may bind to "adaptor molecules," such as Cdc4p, Grr1p, and Skp2p, which may directly contact proteolysis substrates. Two facts argue that F-box proteins are more likely to be further removed from the ubiquination machinery than *SKP1*. First, there are many more F-box proteins than *SKP1*-like genes so far. One would expect more E3 proteins than central components because there are many potential substrates. Secondly, some F-box proteins are likely to be substrates or directly bind substrates, and are therefore furthest removed from the machinery itself. Whether Skp1p acts as a ubiquitin ligase itself or merely directs substrates to Cdc34p or other E2 proteins remains to be determined. Although it shares no sequence homology with known E3 ubiquitin ligases, Skp1p homologs do contain an invariant cysteine, C152 in yeast *SKP1* (Figure 1C). Cysteines are the site of ubiquitin linkage in E1 and E2 proteins. While there is no evidence that Skp1p is regulated, its role in both G1/S and G2/M transitions, like Cdc28p, make it a potential target of cell cycle checkpoint pathways that arrest the cell cycle in response to DNA damage, replication blocks, or spindle defects. It is also possible that Skp1p functions in non–cell cycle pathways, e.g., through the *MET30* gene, and perhaps in nonproteolysis capacities.

Although we have proposed a positive role in promotion of proteolysis, it is formally possible that Skp1p might stabilize other proteins required for proteolysis and thereby function indirectly. This seems unlikely but

remains a possibility. Furthermore, although we have evidence for a role in proteolysis, it is quite possible that in some circumstances Skp1p association with F-box proteins will result in events unrelated to proteolysis.

Ubiquitin-Mediated Proteolysis and Cancer

Zhang et al. (1995) observed increased levels of Skp1p and Skp2p associated with cyclin A/Cdk2 in several transformed cell lines and suggested that the increase in these proteins may contribute to the transformed state. The fact that many transformed cells express higher levels of Skp2p in association with cyclin A may indicate alteration of the ubiquitin-mediated destruction pathway. Since it is clear that this proteolysis pathway is essential for many important cell cycle events, such as chromosome segregation, alteration may enhance genomic instability, as is the case for *skp1* mutants (Connelly and Hieter, 1996). Alternatively, the failure to properly degrade certain cyclins or constitutive degradation of CKIs could directly alter growth control. Recent support for a role for proteolysis in growth control comes from phenotypes of C. elegans *cul-1* mutants. *cul-1* encodes a *CDC53* homolog and mutants in *cul-1* result in additional cell proliferation (Kipreous et al., 1996).

SKP1 is clearly important for several cell cycle events. Each *skp1* mutant phenotype, cell cycle arrest in G1 and in G2, and chromosome instability, potentially could be explained by its role in promoting proteolysis. In addition, its ability to associate with proteins through F-box binding has implicated a new family of genes in ubiquitin-mediated proteolysis. Although the biochemical and genetic analysis of *SKP1* has identified a potentially important component in the control of proteolysis, it raises a number of questions. For example, if Skp1p is recruiting substrates and E3 proteins to the proteolysis machinery, what determines which proteins in the complex are degraded? Also, what is Skp1p contacting in the machinery itself? Do F-box proteins constitute a new family of E3 proteins or proteolysis substrates? Finally, do some tumor cells have defects in ubiquitin-mediated proteolysis of particular substrates? While many of the roles of *SKP1* have not been fully elucidated, the current insight into its function should provide a perspective from which to directly approach these questions.

Experimental Procedures

Media and Yeast Manipulations, Yeast Strains, Plasmids, and Primers

Basic yeast manipulations and media were as described in Rose et al. (1990). Yeast strains and plasmids used in this study are listed in Table 1. Primers used are referred to by letters and have the following sequences:

A, *gacgttataaacaatacaggaatactaacaaacgtagcgcaggattgtactgagagtgca*;
B, *atcatcggaagaaaggatcacagtcatcagaggaaatgtaggctgtgcggtatttcacaccg*;
C, *gaacccatggtgacttctaatgttgtcc*;
D, *gcaaggatcctaacggtcttcagcccattc*;
E, *atacctcgagcaaagtctagtgaaagc*;
F, *gcaaggatccttattgagacgatatctg*;
G, *ggacctaataacgtctttggcttttgaaataagttt*;
H, *aaacttatttcaaaagccaaagacgttattaggtcc*;
I, *tttgaaaattttcaattatgcgcaattcgaggatat*;
J, *atatcctcgaattgcgcataattgaaaattttcaaa*;
K, *tagaaaatctacatcgttggcgaaaaaacttctgat*;
L, *atcagaagtttttttcgccaacgatgtagattttcta*;
M, *ggacctaataacgtctgcggctttgaaataagttt*;
N, *aaacttatttcaaaagccgcagacgttattaggtcc*;
O, *ttttgaaataagtttgaaagctttcaattatttgcaatt*;
P, *aattgcaaataattgaaagctttcaaacttatttcaaaa*;
Q, *taatacgactcactatagggcgaattcgtttttatttttaattttctttcaaatacttccacc atggggtcg tttcccttagctg*;
R, *ggtcatggtattatagttgtc*.

Plasmid Constructions

pCB1, 2, and 3 are plasmid suppressors of *cdc4-1* mutants and correspond to *SKP1*, *CLB4*, and *CDC4*, respectively. The 1.2 kb insert of *SKP1* cDNA was excised as a BamHI–NotI fragment from pCB1 and cloned into BglII–NotI pcDNA3 (Invitrogen) to generate pCB5. A 2.7 kb genomic polymerase chain reaction (PCR) fragment of *SKP1* from 1.2 kb 5′ to 0.8 kb 3′ of the open reading frame was made with primers E and F and cloned into pCRII (Invitrogen), generating pCB12. *SKP1* on a SacI–XbaI fragment from pCB12 was cloned into SacI–XbaI-cleaved pRS415 to generate pCB6.

SKP1 with NcoI at its initiation codon and BamHI at the 3′ end was generated by PCR using primers C and D and cloned into NcoI–BamHI-cleaved pGEX2TKcs (XbaI) to create pCB21, which expresses Gst–Skp1p in bacteria, and into pADH–Gst to generate pCB23 for Gst–Skp1p expression in yeast. pCB31 was generated by cloning SalI–BglII fragment from pCB30 into BamHI–SalI-cleaved pBS II KS(+). For expression of Gst–cyclin F in insect cells, a 3.8 kb XbaI–BglII fragment containing the cyclin F open reading frame fused to Gst was cloned into pVL1393 and virus generated using Baculogold transfer DNA (Pharmingen).

Strain Constructions

Yeast strains are listed in Table 1.

A *skp1::URA3* replacement allele was made by transforming a *URA3* gene flanked by 40 nt of *SKP1* homology on each end by PCR using primer A and primer B and pRS406, into CRY3 to make Y544. *URA3* in Y544 was then replaced by *HIS3* using a *ura3::HIS3* SmaI fragment cleaved from pUH7 (F. Cross, unpublished data) to generate Y545. Y545 containing pCB1 (*SKP1URA3*) were sporulated and germinated on YPGal media. His$^+$ haploids, such as Y546, always contained pCB1 and were sensitive to growth on media containing 5-fluororotic acid (5-FOA).

skp1-11 and *skp1-12* mutant alleles were subcloned by ligating the 2.7 kb KpnI–SacI fragment from pCB8 and pCB9 into KpnI–SacI-cleaved pRS406 to make pCB14 and pCB15. pCB14 and pCB15 were linearized by partial digestion with HindIII and transformed into Y81 selecting for Ura$^+$ colonies and screened by Southern analysis for homologous integration events. These strains were selected on 5-FOA media for excision events and screened for Ts phenotype to generate Y553 (*skp1-11*) and Y555 (*skp1-12*). These strains were crossed to Y80 and sporulated to generate Y552 and Y554.

Generating Conditional SKP1 Alleles

Hydroxylamine mutagenesis of pCB6 was carried out according to Rose et al. (1993). We selected 2×10^4 transformants in Y546 on SC−leucine plates and screened for growth at 37°C by replica-plating onto SC−uracil plates and 5-FOA plates. Four clones showed temperature-sensitive growth on SC−leucine + 5-FOA plates but not on SC−uracil plates. Plasmids were recovered in Escherichia coli and retransformed into Y546 to confirm the Ts phenotype, and two plasmids, pCB7 (*skp1-11*) and pCB8 (*skp1-12*), showed temperature sensitivity and were sequenced to identify the mutations.

Protein Expression

Viruses for Skp1p and Skp2p were from previous studies (Zhang et al., 1995). For protein production, 2×10^6 Sf9 cells were infected with the indicated virus or combination of viruses for 40 hr, at which time cells were labeled with Translabel (ICN) for 4 hr using standard procedures. Lysates were prepared by disrupting cells in 400 μl of NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing 10 mM NaF, 25 mM β-glycerolphosphate, followed by centrifugation (15 min at 14,000 g). Gst-fusion proteins from 100

Table 1. Strains

Strain	Relevant Genotype
Y80	*MATa, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1*
Y81	*MATα, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1*
CRY3	Y80 × Y81
Y543	As Y81 but *cdc4-1*
Y544	As CRY3 but *skp1Δ::URA3/SKP1*
Y545	As CRY3 but *skp1Δ::HIS3/SKP1*
Y546	As Y81 but *skp1Δ::HIS3* + pCB1*(URA3::GAL10-SKP1)*
Y547	As Y80 but *skp1Δ::HIS3* + pCB1*(URA3::GAL10-SKP1)*
Y549	As Y80 but *skp1Δ::HIS3* + pCB23*(LEU2::GST-SKP1)*
Y550	Y81 + pCB23*(LEU2::GST-SKP1)*
Y552	As Y80 but *skp1-11*
Y553	As Y81 but *skp1-11*
Y554	As Y80 but *skp1-12*
Y555	As Y81 but *skp1-12*
Y556	As Y80 but *skp1Δ::HIS3* + pCB6*(SKP1)*
Y557	As Y80 but *skp1Δ::HIS3* + pCB7*(skp1-11)*
Y558	As Y80 but *skp1Δ::HIS3* + pCB8*(skp1-12)*
Y563	As Y80 but *skp1-11 sic1Δ::TRP1*
Y564	*MATa,his3-11,15, leu2-3,-112, trp1, ura3, sic1Δ::TRP1*
YL10	*MATα, cdc34-2, his3Δ, ura3-52, trp1-Δ63, leu2Δ*
MGG12	*MATα, cdc53-1, trp1-1, ura3-52, his3Δ, ade2*
WX131-2c	*MATα, cdc53-2, trp1-7, ura3-52, ade2*

μl of lysate were purified using 10 μl of GSH–Sepharose (4°C for 1 h) and complexes washed three times with 1 ml of binding buffer prior to electrophoresis on 12% SDS–polyacrylamide gels and autoradiography.

Skp1p/Cdc4 Binding

For Skp1p and Cdc4p binding, Gst–Skp1p (pCB23) was expressed in Y80 and extracts prepared as described (Buchman et al., 1988), except that 0.3 M ammonium sulfate was not used. Gst–Skp1p and associated factors were purified using glutathione Sepharose from 2 mg of cell extract. After 3 washes in NETN, bound proteins were resolved in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with antibodies against Cdc4p. For the in vitro binding assay, 1 μg of Gst, Gst–Skp1p, and Gst–Cdc4p expressed in E. coli and bound to glutathione Sepharose beads were incubated for 2 hr with in vitro translated, ^{35}S-labeled protein (TNT Promega) at 4°C in NETN buffer containing protease inhibitors, washed 3× with the same buffer. Bound proteins were resolved using SDS–PAGE and autoradiographed.

Generation of F-Box Point Mutants

A two-step PCR method (94°C for 1 min, 52°C for 45 sec, 72°C for 1.5 min for 30–35 cycles) was used for generating point mutants in *CDC4*. The primers were designed to include an upstream T7 promoter sequence for T7 transcription. The Expand PCR system (Boehringer Mannheim) was used for all PCR reactions. Primers used in addition to primers Q and R were, for the P279A mutation, G and H, for the I286A, O and P, for L290A, I and J, for W316A, K and L, and for LP278-279AA, M and N.

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Table 2. Plasmids

Plasmid	Relevant Markers	Base Vector	Source
pCB1	*GAL-SKP1, URA3, CEN4, ARS1*	pRS316	This study
pCB2	*GAL-CLB4, URA3, CEN4, ARS1*	pRS316	This study
pCB3	*GAL-CDC4, URA3, CEN4, ARS1*	pRS316	This study
pCB5	*SKP1* cDNA	pcDNA3	This study
pCB6	*SKP1, LEU2, CEN4, ARS1*	pRS415	This study
pCB7	*skp1-11, LEU2, CEN4, ARS1*	pRS415	This study
pCB8	*skp1-12, LEU2, CEN4, ARS1*	pRS415	This study
pCB12	*SKP1*	pTA	This study
pCB14	*skp1-11 URA3*	pRS406	This study
pCB15	*skp1-12, URA3*	pRS406	This study
pCB21	*SKP1*	pGEX2TKcs (Xba)	This study
pCB23	*GST-SKP1 LEU2* 2μ	pADHGST	This study
pCB24	*GAL-SIC1 URA3 CEN4, ARS1*	pRS416	This study
pCB30	GAP-cyclin F *URA3* 2μ	pAB23BXN	Bai et al., 1994
pCB31	cyclin F	pBSKSII(+)	Bai et al., 1994
pMT634	*GAL-CLN2 URA3 LEU2*	pRS316	M. Tyers
pMT527	*ADH-CLN2 LEU2*	pRS315	M. Tyers
pWS12933	*GAL-CLB2 URA3*	YCplac133	M. Tyers
pWS945	*GAL-CLB4 URA3*	YCplac133	W. Seufer

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Eradication of pathogenic β-catenin by Skp1/Cullin/F box ubiquitination machinery

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The use of Skp1/Cull 1/F box (SCF) ubiquitin-conjugation machinery as a potential knockout tool offers a means of eradicating disease-causing proteins. Here a chimeric F box protein (CFP) was engineered to achieve selective eradication of pathogenic β-catenin in colorectal cancer. We show that CFP specifically searches for and subsequently links the abnormal β-catenin to the cellular SCF ubiquitination complex. Introduction of the CFP to colorectal cancer cells induced targeted ubiquitination and proteolytic degradation of nuclear and cytoplasmic free β-catenin while preserving its normal cellular adhesion counterpart. Elimination of pathogenic β-catenin suppressed constitutive Wingless/Wnt signaling and inhibited *in vitro* and *in vivo* tumor cell growth. This study demonstrates a practical utility of a SCF-based knockout system as a tool in targeting an abnormal protein that affects growth and transformation.

Abnormal accumulation of protein has catastrophic consequences and is the primary cause of several neuronal and cancer diseases (1–4). β-Catenin, for example, is an essential component of the cell–cell adhesion complex by binding with E-cadherin (5). The protein is also required to activate a variety of TCF4-regulated genes in response to Wnt signaling during embryogenesis (6). In the absence of Wnt signaling, non-E-cadherin-associated free β-catenin is tightly regulated by a multiprotein complex consisting of adenomatous polyposis coli (APC) protein, Axin, and the serine/threonine kinase GSK3β (7–12). The regulation process involves the recognition of β-catenin by APC and subsequent recruitment of Axin and GSK3β. Interaction between these proteins facilitates phosphorylation of β-catenin at its amino-terminal serine residues by GSK3β. After phosphorylation, β-catenin becomes an immediate target for cellular Skp1/Cull 1/F box (SCF) ubiquitination machinery. In human colorectal cancer (CRC), mutations in APC are the earliest detectable genetic events. Loss of APC function causes stabilization and, consequently, high levels of β-catenin accumulation in the nucleus of mutant cells (6). It is believed that nuclear β-catenin binds to TCF4 and activates TCF4 target genes. Constitutive activation of these Wingless/Wnt effectors initiates the development of colorectal neoplasia (14, 15). Although genomic knockout and RNA interference provide powerful reverse genetic tools to study β-catenin by silencing its expression, these technologies lack the ability to discriminate between the abnormal protein and its normal counterpart expressed from the same gene (16, 17). As β-catenin is also required for the normal growth of the cell, targeted cells can not survive when the β-catenin gene is completely silenced with current knockout technologies (18). Targeting proteins to SCF ubiquitination machinery promises an alternative gene-silencing tool (19–21). In this study, we demonstrate that SCF provides a powerful means of eradicating pathogenic β-catenin in CRC. This ability is achieved by engineering a chimeric F box protein (CFP) highly specific in recognizing abnormal β-catenin. We show that introduction of CFP to CRC cells induced targeted ubiquitination and proteolytic degradation of nuclear and cytoplasmic free β-catenin while preserving its cellular adhesion counterpart. Elimination of pathogenic β-catenin suppressed constitutive Wingless/Wnt signaling and inhibited *in vitro* and *in vivo* tumor cell growth.

Materials and Methods

Tissue Culture and Transient Transfection. 293T, HFF, DLD1-tet-off, HCT116-tet-off, LS174-TR, HAβ18, and HAβ85 cells were kindly provided by L. Falo, M. Stinski, B. Vogelstein, Q. Zhan, H. Clevers, and T. Waldman, respectively. BJ fibroblast and colorectal tumor cell lines DLD1 and HCT116 were purchased from American Type Culture Collection. Doxycycline-inducible expression cell lines were constructed as described (22). Transient transfections were performed by using MIRUS *Trans*IT-LT1 reagent (Mirus, Madison, WI). Transfection efficiency was monitored by using an enhanced GFP (EGFP) expression plasmid pTrackEGFP. Cells used for the ubiquitination analysis were cultured in the growth medium containing 12.5 μM proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN; Calbiochem).

Plasmid Clones. pTrackEGFP, pShuttleCMV, pCMV-APC, pTOP-Flash, pFOPFalsh, and pcDNA3F-β-TrCP-linker were kindly provided by B. Vogelstein, H. Clevers, and P. Zhou. pShuttleCMV was slightly modified by inserting a hemagglutinin (HA)-tag and renamed as pShuttleHA. E-cadherin 751–878 and TCF4 1–86 were obtained by PCR amplification of fetal brain cDNA (Clontech). APC 1242-2060, APC 1014-1177, and APCbc, were obtained by PCR amplification of pCMV-APC. F box-containing constructs were obtained by PCR amplification of fetal brain cDNA. The F box–EGFP fusion was constructed by cloning a PCR-amplified EGFP into the *Xho*I–*Xba*I-digested F box constructs. F2APCbc4 and F3APCbc4 were constructed by replacing EGFP with APCbc4. PCR-amplified DNA fragments were confirmed by sequencing and cloned into the pShuttleHA (cloning details and primer sequences for each of the PCR amplification can be obtained on request).

Protein–Protein Interaction, Immunoprecipitation, and Western Blot Analyses. Protein–protein interaction, immunoprecipitation (IP), and Western blot analyses were performed essentially as described (23). For detection of ubiquitinized β-catenin, addition of 12.5 μM ALLN was supplemented to inhibit proteasome activities. The following different primary antibodies were used for IP: anti-HA monoclonal (clone HA-7, Sigma), anti-Flag monoclonal (clone M2, Sigma), anti-E-cadherin monoclonal (Pharmingen), and anti-β-catenin monoclonal (Pharmingen). The antibodies used for Western blot analysis were rabbit anti-Skp1 polyclonal (Zymed), mouse anti-HA monoclonal (clone HA-7, Sigma), mouse anti-Flag monoclonal (Sigma), mouse anti-c-MYC (clone 9E10, Sigma), mouse anti-β-catenin monoclonal (Pharmingen), rabbit anti-α-actin polyclonal (Sigma), rabbit anti-lamin B1 (Zymed), and mouse anti-ubiquitin monoclonal (Santa Cruz Biotechnology). The resulting signals were visualized by exposure to an x-ray film (Sterling, New York). Films were scanned and analyzed for signal strength by IMAGEQUANT software (Molecular Dynamics).

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Abbreviations: SCF, Skp1/Cullin/F box complex; APC, adenomatous polyposis coli; CRC, colorectal cancer; CFP, chimeric F box protein; IP, immunoprecipitation; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; EGFP, enhanced GFP; HA, hemagglutinin.

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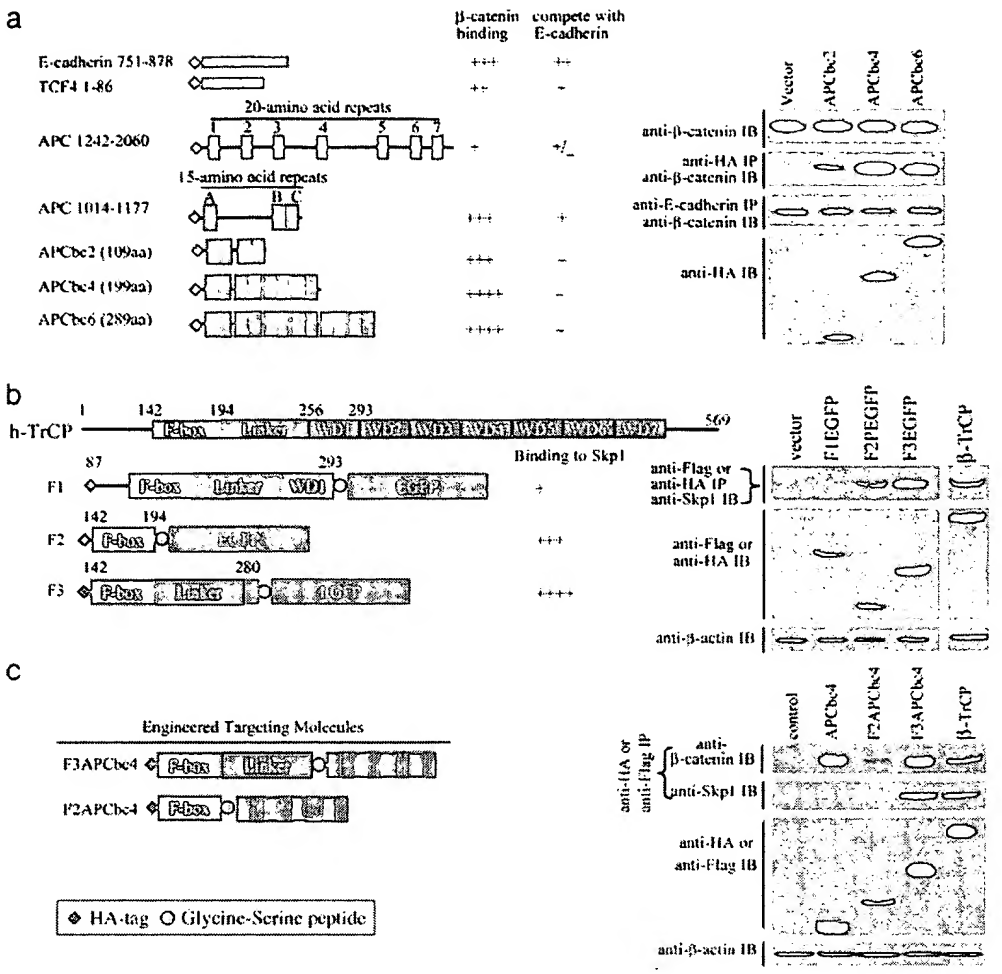

Fig. 1. Generation of CFP-targeting constructs. (*a Left*) Schematic representation of β-catenin-binding motifs expressed in 293T cells and a summary of their ability to bind free β-catenin and the E-cadherin-associated β-catenin. (*a Right*) Coimmunoprecipitation of non-E-cadherin and E-cadherin-associated β-catenin with pShuttleHA (control vector), APCbc2, APCbc4, and APCbc6. Lysates from transfected 293T cells were first immunoprecipitated with anti-HA antibody, and the amount of β-catenin recognized by APCbc2, APCbc4, and APCbc6 was revealed by Western blot analysis with mouse anti-β-catenin antibody (second blot from the top). The anti-HA antibody-cleared protein lysates were subsequently precipitated with anti-E-cadherin antibody, and the amount of β-catenin still associated with E-cadherin was detected by Western blot analysis with mouse anti-β-catenin antibody (third blot from the top). The expression levels of APCbc2, APCbc4, and APCbc6 in the transfected 293T cell lysates are shown on the bottom. Protein lysates with equal amounts of β-catenin from transfected cells were used for the IP (displayed in the top row). (*b Left*) schematic of F box motifs expressed in 293T cells and summaries of their binding abilities to Skp1 protein. (*b Right*) Coimmunoprecipitation of Skp1 protein with F1, F2, and F3. Protein lysates from transfected 293T cells were immunoprecipitated with anti-HA or anti-Flag monoclonal antibody, and the amount of Skp1 protein associated with F1, F2, F3, and β-TrCP was revealed by Western blot analysis with rabbit anti-Skp1 antibody (shown in the top row). The expression levels of F1EGFP, F2EGFP, F3EGFP, and β-TrCP in the transfected cells are shown in the second row. The presence of similar levels of α-actin protein in each lane indicates that an equal amount of protein lysates was used for the IP and Western blot analyses (bottom blot). (*c Left*) Schematic of F2APCbc4 and F3APCbc4 constructs used for transient expression in 293T cells. (*c Right*) Coimmunoprecipitation of Skp1 and β-catenin proteins with APCbc4, F2APCbc4, F3APCbc4, or Flag-tagged β-TrCP. Protein lysates from transfected 293T cells were immunoprecipitated with anti-HA or anti-Flag antibody, and the amount of Skp1 and β-catenin proteins associated with APCbc4, F2APCbc4, F3APCbc4, or β-TrCP was revealed by Western blot analysis with respective antibodies as designated (top and second blots). The expression levels of APCbc4, F2APCbc4, F3APCbc4, or β-TrCP in the transfected 293T cells are shown in the third blot. The presence of similar levels of α-actin protein in each lane indicates that an equal amount of protein lysates was used for the IP and Western blot analyses (bottom row).

Luciferase Assay. Luciferase activity was measured by using the Luciferase Activity Assay kit as described by the manufacturer (Promega).

Cell Fractionation. Cells were fractionated to separate nuclear protein from the cytoplasmic protein by using a NE-PER Nuclear and Cytoplasmic Extraction kit as described by the manufacturer (Pierce).

Immunohistochemistry and Immunofluorescence Staining. Immunohistochemistry and immunofluorescence staining were performed as described (23).

***In Vitro* Cell Growth and Colony Formation Assays.** Cell growth and colony formation assays were performed in 12-well plates essentially as described (24).

***In Vivo* Assays.** Before the injection of any DLD1 tumor cell lines, mice were given drinking water containing doxycycline at a concentration of 200 ng/ml for 3 days. Cells (5×10^6) in the volume of 0.1 ml were inoculated s.c. into 6-week-old female athymic *nu/nu* mice (Charles River Breeding Laboratories) in the right and left flanks. We defined a tumor formation as a xenograft that attained a minimal volume of 200 mm^3 at any point in the experiment.

Results

Generation of CFP-Targeting Construct. To selectively eradicate pathogenic β-catenin in CRC, we designed our targeting molecule based on two principles. First, it should be armed with the selectivity to target only non-E-cadherin-associated free β-catenin. Second, it should destroy seized β-catenin by using SCF ubiquitin-conjugation machinery. To fulfill the first criteria, we have constructed and analyzed a variety of protein motifs known to interact with β-catenin (5, 7–8, 13–15, 25–27). These motifs include 15- and 20-aa repeats of the APC protein; the carboxyl-terminal of E-cadherin; and the amino-terminal domain of TCF4. Each of these motifs was tagged with HA epitope and analyzed for their respective binding affinities to non-E-cadherin-associated β-catenin by transient expression in 293T cells (Fig. 1*a Left*). We defined the selectivity function as those proteins showing minimal competition with E-cadherin-associated β-catenin but maintaining strong binding to free β-catenin in the protein–protein interaction assay. Although all

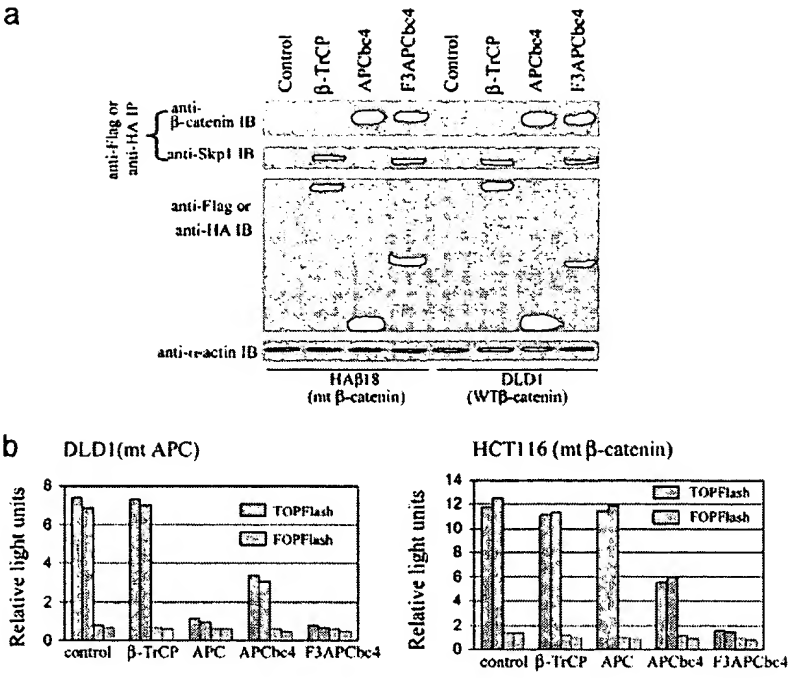

Fig. 2. F3APCbc4 targets both mutant and wild-type β-catenin and suppresses β-catenin/TCF4-mediated transcription activities. (*a*) Coimmunoprecipitation of Skp1, wild-type, or mutant β-catenin, with vector control, Flag-tagged β-TrCP, APCbc4, or F3APCbc4 in CRC cell lines. Protein lysates from transiently transfected HAβ18 (contained a mutant β-catenin allele) and DLD1 (contained wild-type β-catenin but mutant APC) were immunoprecipitated with anti-Flag or anti-HA antibody. Amount of Skp1 or β-catenin protein associated with β-TrCP, APCbc4, or F3APCbc4 was revealed by Western blot analysis with anti-Skp1 or anti-β-catenin antibody (top and second blots). The expression levels of Flag-tagged β-TrCP, APCbc4, or F3APCbc4 in the transfected cells are shown in the third row. The presence of similar levels of α-actin protein in each lane indicates that an equal amount of protein lysates was used for the IP and Western blot analyses (bottom blot). (*b*) Luciferase activities were suppressed in both HCT116 and DLD1 transfected with F3APCbc4 as compared with the vector control, β-TrCP, wild-type APC, and APCbc4. Luciferase activities from two independent transfections are shown.

these protein motifs interacted with β-catenin, the APC 15-aa repeat unit B-C (designated as APCbc), when engineered to contain multiple duplicates, showed the highest affinity to free β-catenin with minimal competition with E-cadherin-associated β-catenin (Fig. 1*a Right*).

To achieve the destroy function, we chose to engineer the F box motif of h-TrCP (28–30). Clones containing F box with deletion of various non-F box sequences were constructed and tagged with HA epitope (Fig. 1*b Left*). The deleted carboxyl-terminal domain of β-TrCP was replaced with EGFP. After the transfection in 293T cells, green fluorescence was visible in three F box-EGFP fusion constructs, namely F1GFP, F2GFP, and F3GFP. Analysis of 293T cell lysates showed that F3EGFP showed levels of expression similar to that of β-TrCP, whereas F1 and F2 fusion proteins appeared to be less stable (Fig. 1*b*). Moreover, the ability of these proteins to form a complex with SCF was determined by their physical interactions with Skp1 (28). Biochemical analysis demonstrated that both F2 and F3 interacted with Skp1, whereas F1 showed almost no interaction. The levels of interaction between F3 and Skp1 appeared to be similar to that of β-TrCP (Fig. 1*b Top Right*). In contrast, F2 only had a weak interaction with Skp1.

To combine the selectivity and the destroy functionalities into a full-length CFP molecule, APCbc4, a construct with four APCbc repeat units, which showed the strongest affinity to β-catenin, was selected to accomplish the "selectivity" function. A 10-aa serine-glycine peptide was used to connect APCbc4 to F2 or F3 (Fig. 1*c Left*). The full-length CFP molecules, F2APCbc4 and F3APCbc4, were transiently expressed in 293T cells. β-TrCP and APCbc4 were used as a control. Analysis of protein lysates from transient transfected 293T cells demonstrated that F3APCbc4 interacted with both β-catenin and Skp1 proteins on a level similar to that of β-TrCP (Fig. 1*c Right*). In contrast, APCbc4 interacted only with β-catenin. Although F2APCbc4 retained a weak interaction with β-catenin, it did not interact with Skp1. It is possible that combination of F2 with APCbc4 resulted in an incorrect protein folding and consequently affected the stability and function of F2APCbc4 (Fig. 1*c Right*).

CFP Targets Both Mutant and Wild-Type β-Catenin to the SCF Complex and Suppresses β-Catenin/TCF4-Regulated Transcription Activity. To evaluate the ability of CFP in targeting both mutant and wild-type β-catenin to the SCF complex, F3APCbc4, APCbc4, or β-TrCP was introduced into two CRC cell lines, HAβ18 and DLD1. DLD1 has mutations in APC, whereas HAβ18 contains only mutant β-catenin as it is derived from HCT116 and has a knockout in wild-type β-catenin allele (16). IP analysis of protein lysates from transiently transfected HAβ18 and DLD1 cells showed that F3APCbc4 had the ability to connect both mutant and wild-type β-catenin to SCF (Fig. 2*a*). In contrast, APCbc4, although recognizing both mutant and wild-type β-catenin, did not interact with SCF. On the other hand, β-TrCP, although interacting with Skp1, did not recognize β-catenin (Fig. 2*a*).

To determine the biological effect, a reporter system, pTOPFlash and pFOPFlash, was used to measure β-catenin/TCF4-mediated transcription activities in CRC cell lines HCT116 and DLD1 (13). Each of the reporters was cotransfected with vector control, APCbc4 or F3APCbc4, into HCT116 or DLD1 cells. Tumor suppressor APC and native F box protein β-TrCP were used in the transient transfection as a control. Analysis of reporter activities showed that β-catenin/TCF4-mediated transcription activity in both CRC cell lines were significantly suppressed by F3APCbc4 (Fig. 2*b*). Consistent with published observations, APC suppressed β-catenin/TCF4-mediated transcription activity in DLD1, but not in HCT116 cells (14, 15). Moreover, β-TrCP showed little effect on β-catenin/TCF4-mediated transcription activity. APCbc4, on the other hand, demonstrated a slight effect on β-catenin/TCF4-mediated activity, likely through its competition with TCF4 for the free β-catenin.

CFP Selectively Targets Pathogenic β-Catenin Through Ubiquitin-Conjugated Proteolysis. To determine whether the suppression of β-catenin/TCF4 transcriptional activities by F3APCbc4 was mediated by targeted ubiquitination of β-catenin as designed, we attempted to establish a doxycycline-inducible expression of APCbc4 and F3APCbc4 in both HCT116 and DLD1 cell lines. Experiments to establish a stable doxycycline-inducible F3APCbc4 cell line in HCT116 proved to be problematic because clones generated from HCT116 cells were unstable. This instability may likely be caused by the hypermutable nature of HCT116 cells (31). However, inducible expression of F3APCbc4 and its minus F box control

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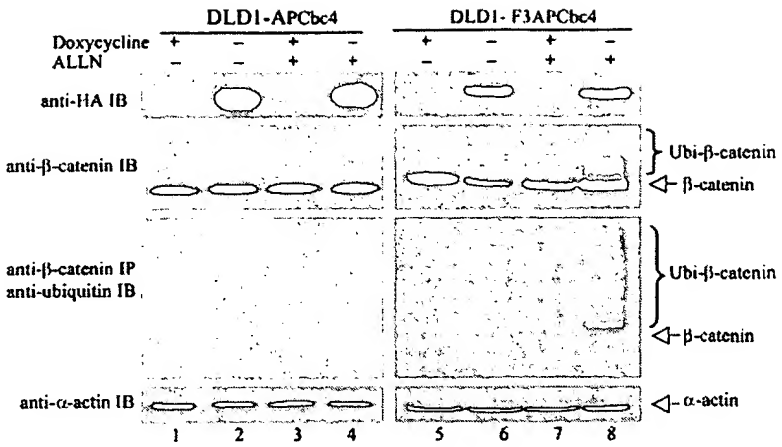

Fig. 3. F3APCbc4-mediated linkage of abnormal β-catenin to ubiquitin-conjugation machinery. IP and Western blot analyses of DLD1-APCbc4 and DLD1-F3APCbc4 show F3APCbc4 (clone F5)-mediated ubiquitination and degradation of pathogenic β-catenin in CRC cells. Reduction of β-catenin in CRC cells mediated by F3APCbc4, but not by APCbc4, is shown in the second row (compare lanes 3, 4, 5, and 6). For detection of F3APCbc4-mediated ubiquitination of β-catenin in CRC cells, DLD1-APCbc4 or DLD1-F3APCbc4 was cultured in the presence of 12.5 μM ALLN for 12 h under the induced or uninduced condition. The second row shows that ubiquitinized β-catenin is present in DLD1-F3APCbc4 but not in DLD1-APCbc4. The presence of ubiquitin-conjugated β-catenin is further demonstrated when the same protein lysates were immunoprecipitated with anti-β-catenin monoclonal antibody and the amount of ubiquitinized β-catenin was probed with anti-ubiquitin antibody (third blot as designated). The top row shows levels of APCbc4 or F3APCbc4 under induced or noninduced conditions. Levels of α-actin in the bottom row indicate that equal amounts of protein lysate were used for the IP and Western blot analyses.

APCbc4 were readily established in DLD1. To determine the biological effect of F3APCbc4 in DLD1 cells, APCbc4 and F3APCbc4 clones responsive to doxycycline-induced expression were selected. When F3APCbc4 expression was induced for 24 h, a significant reduction in β-catenin level was observed (Fig. 3, second blot, compare lanes 5 and 6). In contrast, this reduction was not observed in DLD1-APCbc4 (Fig. 3, lanes 1 and 2). To determine whether reduced β-catenin level was mediated by targeted ubiquitination, ALLN, a potent proteasome inhibitor, was used to stabilize the ubiquitinized β-catenin. Analysis of protein lysates from induced CRC cells treated with ALLN showed accumulation of ubiquitinized β-catenin in DLD1-F3APCbc4, whereas such an accumulation was not evident in DLD1-APCbc4 (Fig. 3, second blot, lanes 4 and 8). To confirm this result, the same protein lysates were immunoprecipitated with anti-β-catenin antibody. The immunoprecipitated proteins were probed with anti-ubiquitin antibody to reveal ubiquitinized β-catenin. The analyses showed that ubiquitin-conjugated β-catenin was present only in induced DLD1-F3APCbc4 treated with ALLN, whereas it was not observed in the noninduced or in the control DLD1-APCbc4 (Fig. 3, third blot, lanes 3, 4, 7, and 8).

Targeted Destruction of Nuclear β-Catenin Suppresses Wingless/Wnt Signaling. Nuclear β-catenin is the prime cause of constitutive β-catenin/TCF4-mediated Wingless/Wnt signaling activities in CRC cells. To examine whether nuclear β-catenin was a key target of CFP, nuclear and cytoplasmic fractions from DLD1-APCbc4 and DLD1-F3APCbc4 were analyzed. To ensure that no crosscontamination occurred between the nuclear and cytoplasmic fractions, a nuclear membrane-associated molecule, lamin B1, was used as a reference. Protein lysates from normal human foreskin fibroblast (HFF) cells were included as a control. High levels of nuclear β-catenin were observed in the parental DLD1-tet and DLD1-F3APCbc4, but not in HFF. After the induction by removing doxycycline from the culture medium, F3APCbc4 became detectable at 9 h and reached a steady state at 12 h, whereas APCbc4 continued to accumulate (Fig. 4*a*, top blot). Reduction in nuclear β-catenin was noticeable at 9 h after the induced expression of F3APCbc4 (Fig. 4*a*, second blot, lane 7). Significant reduction in nuclear β-catenin was observed in 24 h (Fig. 4*a*, lane 9). After 48 h, nuclear β-catenin was virtually eliminated from DLD1-F3APCbc4 (Fig. 4*a*, lane 10). In contrast, no changes in the nuclear β-catenin level were observed in DLD1-APCbc4 throughout the entire time course; indicating that APCbc4 without F box was incapable of inducing β-catenin destruction (Fig. 4*a Left*, second blot). To provide evidence that the reduction in nuclear β-catenin was associated with F3APCbc4-mediated ubiquitination, DLD1-APCbc4 and DLD1-F3APCbc4 were cultured in the presence of proteasome inhibitor ALLN under the induced or uninduced condition. Protein lysates from these cells were harvested at each designated induction time and analyzed for the presence of ubiquitinized β-catenin. Concurrent with the appearance of F3APCbc4 after its induction in CRC cells, ubiquitinized β-catenin was detected as early as 9 h in DLD1-F3APCbc4, but not in DLD1-APCbc4 (Fig. 4*a*, bottom blot). Although the nuclear β-catenin was targeted by F3APCbc4, the cytoplasmic β-catenin levels remained largely unaffected in DLD1-F3APCbc4 (Fig. 4*a*, second blot from the bottom). Consistent with this observation, immunofluorescence staining revealed that, although the staining of nuclear β-catenin disappeared in 24 h after induced expression of F3APCbc4, the membrane E-cadherin-bound β-catenin did not (Fig. 4*b*). Taken together, these results suggested that F3APCbc4 specifically targeted nuclear β-catenin for ubiquitination.

c-MYC was a crucial target for β-catenin/TCF4-mediated Wingless/Wnt signaling activities. DLD1 had high levels of nuclear c-MYC protein (32). We therefore examined the effect of F3APCbc4 on c-MYC expression. Biochemical analysis of nuclear fraction from DLD1-F3APCbc4 showed that reduction of nuclear c-MYC level became evident at 12 h after the induction (Fig. 4*a*, third blot, lane 8). Significant reduction of c-MYC level was observed after 24 h (Fig. 4*a*, third blot, lane 9). When nuclear β-catenin was eliminated, c-MYC expression was blocked in DLD1-F3APCbc4 (Fig. 4*a*, third blot, lane 10). Slight reduction of c-MYC level was also noticeable in DLD1-APCbc4, presumably as the result of competition between APCbc4 and TCF4 for the free β-catenin (Fig. 4*a*, third blot, lanes 1–5). Nonetheless, the effect was not significant compared with that of F3APCbc4.

Eradication of Pathogenic β-Catenin Inhibited CRC Cell Growth *in Vitro* and *in Vivo*. It was evident that the growth of CRC cells was immediately suppressed and, consequently, that the CRC cells died after the induction of F3APCbc4 *in vitro* (Fig. 5*a Right*). In addition, the ability of CRC cells to colonize in a collagen gel was completely suppressed by induced expression of F3APCbc4 (Fig. 5*b Bottom*). Furthermore, an *in vivo* tumorigenicity assay demonstrated that a rapid tumor regression in *nu/nu* mice was accompanied by the induction of F3APCbc4 when doxycycline was removed from the drinking water (Table 1). In contrast, tumors continued to grow in the control nude mice without the induced expression of F3APCbc4. Moreover, APCbc4 had no significant effect on the *in vitro* growth of CRC cells and did not show any effect on *in vivo* tumor growth (Fig. 5 *a Left* and *b* and Table 1). These results suggested that eradication of pathogenic β-catenin mediated by CFP molecule F3APCbc4 was responsible for the inhibitory effect on the *in vitro* and *in vivo* growth of CRC.

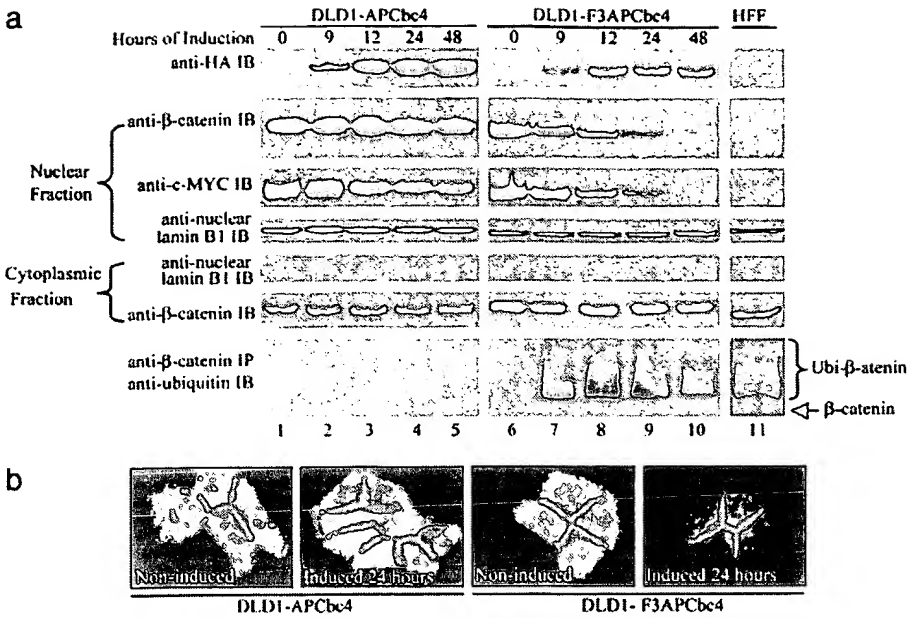

Fig. 4. Targeted destruction of pathogenic β-catenin suppresses Wingless/Wnt signaling. (*a*) IP and Western blot analyses show the reduction of nuclear β-catenin and suppression of c-MYC mediated by F3APCbc4 in CRC cells. The expression level of APCbc4 or F3APCbc4 after the removal of doxycycline for the indicated times is shown in the top blot. Levels of nuclear β-catenin reduction and subsequent suppression of c-MYC associated with induced expression of APCbc4 or F3APCbc4 are shown in the second and third blots. Nuclear fraction from HFF cells was used as control (lane 11). The presence of similar levels of nuclear lamin B1 protein in the nuclear fraction indicates that equal amounts of nuclear lysates were used for the Western blot analysis (fourth blot). Western blot analysis of the cytoplasmic fraction shows that the level of membrane E-cadherin-associated β-catenin was not affected by F3APCbc4 (second blot from the bottom). Absence of nuclear lamin B1 protein in the cytoplasmic fraction indicates that no crosscontamination occurred between the nuclear and cytoplasmic fractions (third blot from the bottom). For detection of F3APCbc4-mediated ubiquitination of β-catenin in CRC cells, DLD1-F3APCbc4 and DLD1-APCbc4 were cultured in the presence of 12.5 μM ALLN for 6 h under induced or uninduced conditions. The bottom blot shows the presence of ubiquitinized β-catenin in CRC cells associated with the induced expression of F3APCbc4 (lanes 7–10) but not with that of APCbc4 (lanes 2–5). HFF cells were included as a positive control (lane 11). (*b*) Immunofluorescence staining shows the disappearance of nuclear and cytoplasmic β-catenin but not the E-cadherin-associated β-catenin in DLD1-APCbc4. No changes in nuclear or cytoplasmic β-catenin are evident in DLD1-APCbc4.

Discussion

The ubiquitin-conjugation machinery is an elaborate mechanism responsible for the destruction of a variety of unwanted proteins, which arise either from misfolding, oxidization, or from normal cycling proteins involved in cell cycle and signaling (1, 33, 34). These features have prompted several earlier studies to explore SCF ubiquitin-conjugation machinery as a possible protein knockout tool (19–21). One study demonstrated SCF-mediated degradation of retinoblastoma protein (20). A very recent study showed that an artificially designed "Protacs" effectively eradicated a targeted methionine aminopeptidase 2 protein (21). The results of our experiments provide further evidence demonstrating that gene silencing using cellular ubiquitin-conjugation machinery is highly effective in eradicating a disease-causing protein. Such a level of efficiency was required to suppress Wingless/Wnt signaling effectively and to induce tumor cell death *in vitro* and *in vivo*. F3APCbc4-mediated tumor suppression was the direct result of targeted degradation of nuclear β-catenin. We noticed that β-catenin ubiquitination occurs concurrently with induced expression of F3APCbc4. Despite high levels of β-catenin accumulation in CRC cells, significant reduction in pathogenic β-catenin was achieved in 24 h and nuclear β-catenin was completely eliminated from CRC cells within 48 h after the induction of F3APCbc4. Elimination of nuclear β-catenin had a dramatic effect on the growth of CRC cells both *in vitro* and *in vivo*. CRC cells were immediately arrested and lost the ability to form colonies in a collagen gel. The growth-inhibitory effect mediated by CFP seemed to be more potent than that of native tumor suppressor APC, since APC-mediated CRC cell death occurred significantly slower (35). It is conceivable that F3APCbc4 accomplishes this effect by squelching the SCF component. Nevertheless, squelching of the SCF component alone was not sufficient to inhibit the growth of CRC cells because F3EGFP- or EGFP-tracked β-TrCP was unable to mediate growth suppression in DLD1 (Y.S. and B.L., unpublished observations). The potent growth-inhibitory effect mediated by CFP is likely due to its high efficiency in recruiting pathogenic β-catenin directly to the SCF-ubiquitination machinery without the need for APC-mediated

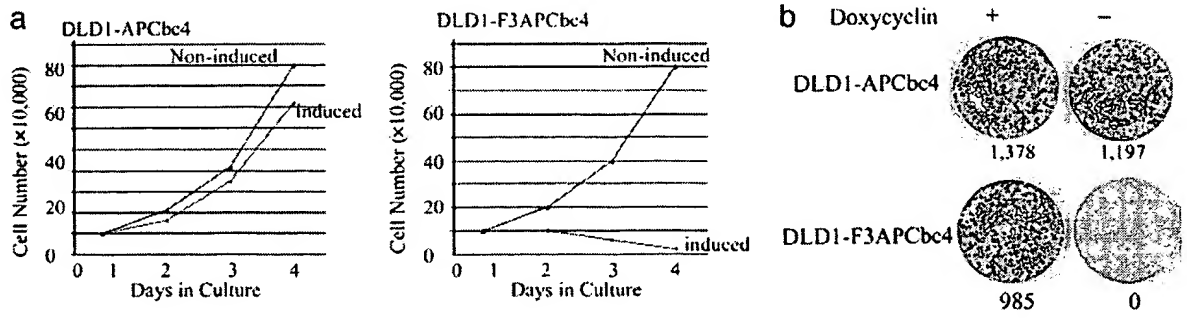

Fig. 5. Targeted destruction of pathogenic β-catenin inhibits the growth of DLD1 cells *in vitro* and *in vivo*. (*a*) Growth kinetics of CRC cells with or without induced expression of APCbc4 (growth chart on the left) or F3APCbc4 (growth chart on the right). Numbers were derived from an average of the results of three different culture dishes. (*b*) Colony formation in collagen gel. Proliferation was inhibited in DLD1 by F3APCbc4 but not by APCbc4. This proliferation was visualized by crystal violet staining of *in vitro* cultured DLD1 cells with or without induced expression of APCbc4 or F3APCbc4 for 9 days until the individual colonies were visible. Numbers of colonies are illustrated.

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Table 1. Tumor suppression in immunodeficient nude mice

Tumor cells	Induction	No. of tumors/ no. of injections
DLD1-F3APCbc4	No	12/12
DLD1-F3APCbc4	Yes	0/12
DLD1-APCbc4	No	12/12
DLD1-APCbc4	Yes	12/12

Cells (5×10^6) were inoculated in the right and left flanks. When the xenograft were attained a minimal volume of 200 mm^3, half the mice with a tumor were fed with drinking water free of doxycycline to induce F3APCbc4 or APCbc4 expression in tumor cells. Tumor suppression was defined as any xenograft that regressed from 200 mm^3 to no measurable tumor volumes in 2 weeks after the induction of F3APCbc4.

phosphorylation. Moreover, CFP molecule F3APCbc4 has the ability to target both wild-type and mutant β-catenin in CRC cells, whereas APC is only capable of targeting wild-type β-catenin. Finally, we found that introduction of the APCbc4 domain alone to the DLD1 cells, while slightly reducing β-catenin/TCF4-mediated transcription activities and the associated cell growth rate through its competition with TCF4 for β-catenin binding, was insufficient to induce DLD1 cell death *in vitro* and *in vivo* (36). Collectively, these results indicate that pathogenic β-catenin is likely required for the maintenance of tumor, as none of the established tumors can survive in nude mice when non-membrane-associated β-catenin was selectively eradicated by induced expression of CFP molecule. Our results support a hypothesis that mutated oncogenes associated with the genesis of cancer are also required for tumor maintenance as demonstrated in c-MYC and Ras (37–39).

An obvious advantage of the SCF-based protein-knockout system is that it could be used as a general method for selectively eradicating a subset of a targeted protein. This selective eradication can be accomplished through incorporation of a subcellular localization signal or a specific interaction domain to a CFP molecule. Our CFP molecule, although specifically designed to block Wingless/Wnt signaling in CRC, could be refitted to target other disease-causing proteins. The β-catenin-recognition domain, APCbc4, can be replaced by a motif known to recognize the other targeted protein. A recognition motif could be selected from a natural partner or a regulator of the targeted protein. When such a natural partner or regulator is not known, the recognition motif could also be obtained through screening in a yeast two-hybrid system, a random peptide library, or an antibody against the disease-causing protein (40, 41). In addition, a recognition motif can often be reengineered to maximize its specificity and binding affinity to a targeted protein as we did for the APCbc-binding motif. Finally, an effective CFP-targeting molecule is expected to follow the same rule as the native β-TrCP, which involves the recruitment and productive positioning of the substrate relative to the E2 active site for ubiquitination (42). In our case, retaining the native linker between the F box and substrate recognition domain was a critical factor. Although the exact linker sequences required for maintaining the structural stability or correct protein folding have to be tested, recent studies of Cdc4-Skp1 or β-TrCP1-Skp1-β-catenin crystal structures have unveiled the critical sequences involved in positioning the substrate relative to the rest of SCF complex (42, 43).

Unlike genomic knockout or RNA interference, which completely silence expression of the entire gene, the SCF-based knockout system can reveal gene function by perturbing different function domains or cellular localization. This ability is obviously advantageous in the study of multifunctional proteins involved in the growth and development.

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Targeted degradation of β-catenin by chimeric F-box fusion proteins

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Abstract

Adenomatous polyposis coli (APC) tumor suppressor protein, together with Axin and glycogen synthase kinase 3β (GSK-3β), forms a Wnt-regulated signaling complex that mediates phosphorylation-dependent degradation of cytoplasmic β-catenin by ubiquitin-dependent proteolysis. Degradation of phosphorylated β-catenin is initiated by interaction through the WD40-repeat of a F-box protein β-TrCP, a component of SCF ubiquitin ligase complex. Mutations in APC, Axin, and β-catenin that prevent down-regulation of cytoplasmic β-catenin are found in various types of cancers. In the search for efficient treatment and prevention of malignancies associated with increased levels of cytoplasmic β-catenin, we created chimeric F-box fusion proteins by replacing the WD40-repeat of β-TrCP with the β-catenin-binding domains of Tcf4 and E-cadherin. Expression of chimeric F-box fusion proteins successfully promotes degradation of β-catenin independently of GSK-3β-mediated phosphorylation. More importantly, this degradation does not require intact APC protein (pAPC).
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Keywords: β-Catenin; F-box; β-TrCP; Targeted degradation

β-Catenin is a multifunctional protein that plays an essential role in the transduction of Wnt signals [1,2]. It is also a component of the cadherin cell adhesion complex. In the absence of Wnt signaling, the cytoplasmic level of β-catenin is kept low through interaction with a protein complex composed of APC, Axin, protein phosphatase 2A (PP2A), and glycogen synthase kinase 3β (GSK-3β). In this complex, β-catenin is phosphorylated by GSK-3β [1,2]. Phosphorylation of β-catenin results in its ubiquitination, mediated by β-TrCP in combination with Skp1 and Cullin1, and subsequent degradation through a proteasome-dependent pathway [1,2]. β-TrCP contains two essential modular domains. One is the F-box region containing a ~40 amino-acid motif responsible for binding Skp1 and forming the Skp1/Cullin1/F-box protein$^{\beta TrCP}$ ($SCF^{\beta TrCP}$) ubiquitin-conjugating complex. The other is a region containing WD40-repeats responsible for binding phosphorylated β-catenin. β-TrCP thus serves as an intracellular receptor for phosphorylated β-catenin, and in combination with Skp1 and Cullin1, facilitates ubiquitination reaction between ubiquitin-conjugating enzymes and β-catenin by bringing them together. β-TrCP also binds phosphorylated IκB and Vpu through the WD40-repeat to mediate subsequent ubiquitination and degradation [3].

Activation of Wnt signaling leads to the inactivation of GSK-3β, resulting in accumulation of cytoplasmic β-catenin. At elevated cytoplasmic levels, β-catenin translocates to the nucleus and cooperates with a member of the T-cell factor (Tcf)/lymphocyte enhancer-binding factor (Lef) family to activate expression of target genes, such as c-Myc and cyclin D1 [1].

Mutational inactivation of APC initiates most colorectal carcinomas. Several lines of evidence indicate that the tumor suppressor activity of the pAPC (APC protein) relies, at least in part, on its ability to bind and subsequently down-regulate cytoplasmic β-catenin [4]. Somatic point mutations in the amino-terminal β-catenin that alter putative GSK-3β phosphorylation residues have been reported in various types of cancers [5]. These mutations weaken or abolish the association of β-catenin with β-TrCP and lead to an accumulation of stable

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cytoplasmic β-catenin. Mutations in Axin that prevent GSK-3β-mediated phosphorylation and subsequent down-regulation of cytoplasmic β-catenin are also found in hepatocellular carcinomas [6] and colorectal cancers [7], implicating β-catenin as a major etiologic target of APC in carcinogenesis. To seek a new method to efficiently treat and prevent malignancies associated with increased levels of cytoplasmic β-catenin, we created chimeric F-box fusion proteins by replacing the WD40-repeat with the β-catenin-binding domains of Tcf4 and E-cadherin. Here we report that expression of chimeric F-box fusion proteins can successfully promote degradation of β-catenins in a manner independent of both GSK-3β-mediated phosphorylation and the presence of intact pAPC.

Materials and methods

Plasmids. The Flag-tagged expression constructs were amplified and inserted into pcDNA3 (Invitrogen) using standard PCR cloning techniques. All constructs described in this manuscript were verified by DNA sequencing. Plasmids expressing Myc-tagged β-catenin and HA-tagged ubiquitin were kindly provided by Drs. P. Polakis and D. Bohmann, respectively. Flag-tagged wild-type and mutant β-catenins are described elsewhere [8].

Cell culture, transfection, reporter assay, immunoprecipitation, and immunoblot analysis. 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Colon cancer cell line SW480 was maintained in Leibovitz's L-15-10% FBS. DNA transfection experiments were carried out by calcium phosphate method or FuGene (Roche) method according to manufacturer's instructions. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Tcf/Lef reporter assay was performed as described before [8]. Briefly, subconfluent cells were co-transfected with a reporter construct (pTOPFLASH or pFOPFLASH), an internal control, and indicated plasmids in six-well plates. Luciferase and β-galactosidase activity was measured 24 h after transfection. Co-immunoprecipitation was also performed as previously described [8]. Briefly, cells were lysed in RIPA buffer (0.05 M Tris buffer, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS) supplemented with protease inhibitors. Prior to immunoprecipitation, lysates were precleared with mouse IgG and protein G–agarose. Flag-tagged proteins were immunoprecipitated from supernatants by mouse anti-Flag monoclonal antibody (M2, Sigma) conjugated to agarose (Santa Cruz Biotechnology). For immunoblotting, cells were lysed directly in Laemmli–SDS sample buffer 48 h after transfection. Total cellular proteins (40 μg/lane) were separated by 4–20% gradient Tris–glycine SDS–PAGE (Invitrogen) and transferred to nitrocellulose membrane. Proteins were detected with primary antibodies and horseradish peroxidase-conjugated secondary antibodies using an ECL system (Amersham) and PhosphorImager (Molecular Dynamics). Primary antibodies used were mouse anti-c-Myc monoclonal antibody (9E10, Santa Cruz Biotechnology), mouse anti-FLAG monoclonal antibody (M2, Sigma), mouse anti-HA monoclonal antibody (HA.11, Babco), mouse anti-GFP monoclonal antibody (Clontech), mouse anti-β-catenin monoclonal antibody (C-19220, Transduction Laboratory), and rabbit anti-Skp1 polyclonal antibody (H-163, Santa Cruz Biotechnology). Blots were analyzed with a Lumi-Imager (Boehringer–Mannheim).

Pulse-chase analysis. To perform pulse-chase analysis of ectopically expressed Myc-tagged β-catenin, 239T cells were transiently transfected in six-well plates. Thirty-six hours after transfection, cells were pulse-labeled for 1 h with 0.1 mCi [^{35}S]methionine and -cysteine per each well and then chased with cold media. Cells were lysed in RIPA buffer supplemented with protease inhibitors at the indicated times. After centrifugation of the lysates, Myc-β-catenin was immunoprecipitated from supernatants by mouse anti-c-Myc monoclonal antibody (9E10) conjugated to agarose (Santa Cruz Biotechnology). After three washes with RIPA buffer, immunoprecipitates were subjected to SDS–PAGE. Dried gels were analyzed with a PhosphorImager (Molecular Dynamics).

Immunofluorescence microscopy. Cells were fixed 36 h post-transfection and double-labeled for FLAG-tagged proteins using an anti-Flag antibody (M2, Sigma) and anti-endogenous β-catenin antibodies (rabbit IgG, clone C-2206, 1:2000, Sigma) using a standard indirect immunofluorescence staining method.

Results

Chimeric F-box fusion proteins interact with endogenous β-catenin and Skp1

We created two FLAG-tagged chimeric F-box fusion proteins, Fbox-βBD^{Tcf4} and Fbox-βBD^{Ecad}, by replacing the WD40-repeat of β-TrCP with the β-catenin-binding domains (βBD) from Tcf4 (amino-acid 1–100, βBD^{Tcf4}) or E-cadherin (amino-acid 800–882, βBD^{Ecad}) [9] (Figs. 1A and B). Both βBD^{Tcf4} and βBD^{Ecad} bind the central region of β-catenin consisting of 12 tandem copies of a 42 amino-acid sequence known as the armadillo repeat [9]. We also created Flag-tagged constructs expressing full length β-TrCP (β-TrCP), β-TrCP lacking the WD40-repeat (F-box), βBD^{Tcf4} and βBD^{Ecad} (Figs. 1A and B).

Since F-box-containing proteins serve as receptors for specific substrates forming the common core Skp1/Cullin/F-box protein (SCF) machinery to bridge the association between the E2 ubiquitin-conjugating enzymes and substrates to facilitate ubiquitin transfer, we first tested whether our FLAG-tagged constructs interact with endogenous β-catenin and Skp1, a component of the SCF complex. As shown in Fig. 2, Fbox-βBD^{Tcf4} and Fbox-βBD^{Ecad} pulled down both β-catenin and Skp1, suggesting that these artificial chimeric F-box fusion proteins can bind both β-catenin and SCF machinery similar to β-TrCP. Fbox or βBDs alone can only bring down either Skp1 or β-catenin, respectively.

Down-regulation of β-catenin by chimeric F-box-fusion proteins

Next, we co-expressed Myc-tagged β-catenin in 293T cells in combination with a FLAG-tagged Fbox-βBD^{Tcf4}, Fbox-βBD^{Ecad}, Fbox, βBD^{Tcf4} or βBD^{Ecad} expression construct. As shown in Fig. 3A, expression of Fbox-βBD^{Tcf4} or Fbox-βBD^{Ecad} led to a dramatic decrease in the amount of Myc-β-catenin. On the other hand, expression of Fbox, βBD^{Tcf4}, or βBD^{Ecad} did not show significant effects. Furthermore, overexpression of chimeric F-box fusion proteins did not affect the amount of IκB, an endogenous substrate of β-TrCP (data not shown),

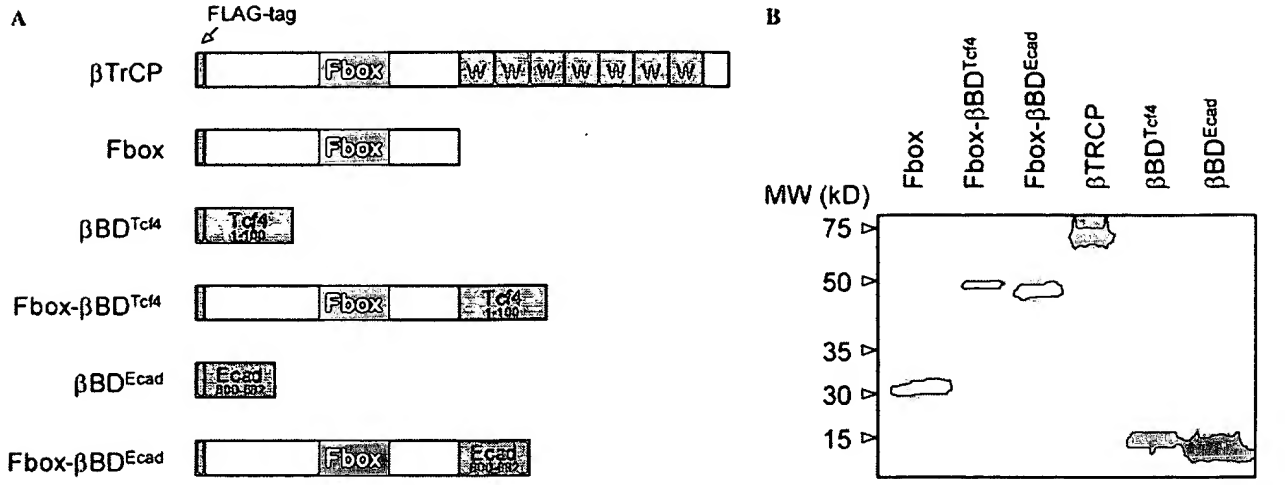

Fig. 1. Structure and expression of chimeric F-box fusion proteins. (A) Schematic representation of chimeric F-box fusion proteins. (B) Expression of FLAG-tagged constructs. 293T cells were transiently transfected with plasmids expressing indicated constructs and labeled with [^{35}S]methionine and-cysteine. Expressed proteins were immunoprecipitated from whole cell extracts using an anti-FLAG antibody and subjected to SDS–PAGE followed by autoradiography.

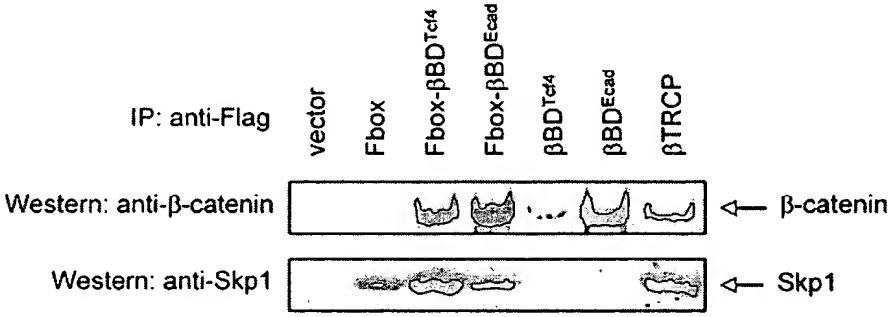

Fig. 2. Chimeric F-box fusion proteins interact with endogenous β-catenin and Skp1. 293T cells were transfected with a plasmid expressing FLAG-tagged Fbox, Fbox-βBD^{Tcf4}, Fbox-βBD^{Ecad}, βBD^{Tcf4}, βBD^{Ecad}, or β-TrCP as indicated. The whole cell extract (WCE) was immunoprecipitated using an anti-FLAG antibody and associated endogenous β-catenin and Skp1 was detected by immunoblotting using an anti-β-catenin antibody and an anti-Skp1 antibody, respectively.

suggesting that introduction of chimeric F-box fusion proteins has little interference effect on the endogenous SCF proteolytic activity.

Interestingly, the F-box domains of F-box proteins are almost always located in the amino-terminus side of the protein-interacting domains. The chimeric constructs containing the F-box domain down-stream of the β-catenin-binding domain did not down-regulate as effective as chimeric constructs containing the F-box domain upstream of the β-catenin-binding domain (data not shown).

The effect of F-box fusion proteins on β-catenin stability was further examined in pulse-chase experiments. Myc-tagged β-catenin was co-expressed with vector, β-TrCP, Fbox-βBD^{Tcf4}, or Fbox-βBD^{Ecad} in 293T cells and the rate of Myc-β-catenin turnover was examined. As shown in Fig. 3B, co-expression with Fbox-βBD^{Ecad} or Fbox-βBD^{Tcf4} both significantly reduced the half-life of Myc-β-catenin. Interestingly, the half-life of Myc-β-catenin was not affected by co-transfected β-TrCP, suggesting that the endogenous β-TrCP may be sufficient to facilitate the degradation of phosphorylated β-catenin. These results demonstrate that chimeric F-box fusion proteins can promote down-regulation of β-catenin in a post-translational manner.

To confirm that the degradation of β-catenin by Fbox-βBD^{Tcf4} and Fbox-βBD^{Ecad} was ubiquitin-dependent, we transfected 293T cells with Myc-tagged β-catenin, HA-tagged ubiquitin in combination with vector, F-box, Fbox-βBD^{Tcf4} or Fbox-βBD^{Ecad}. As shown in Fig. 3C, overexpression of Fbox-βBD^{Tcf4} or Fbox-βBD^{Ecad} significantly increased the ubiquitination of β-catenin. As expected, negative controls using control antibodies did not show non-specific pulldown in the immunoprecipitation reactions.

Taken together, these results indicate that the SCF machinery can be directed toward the degradation of

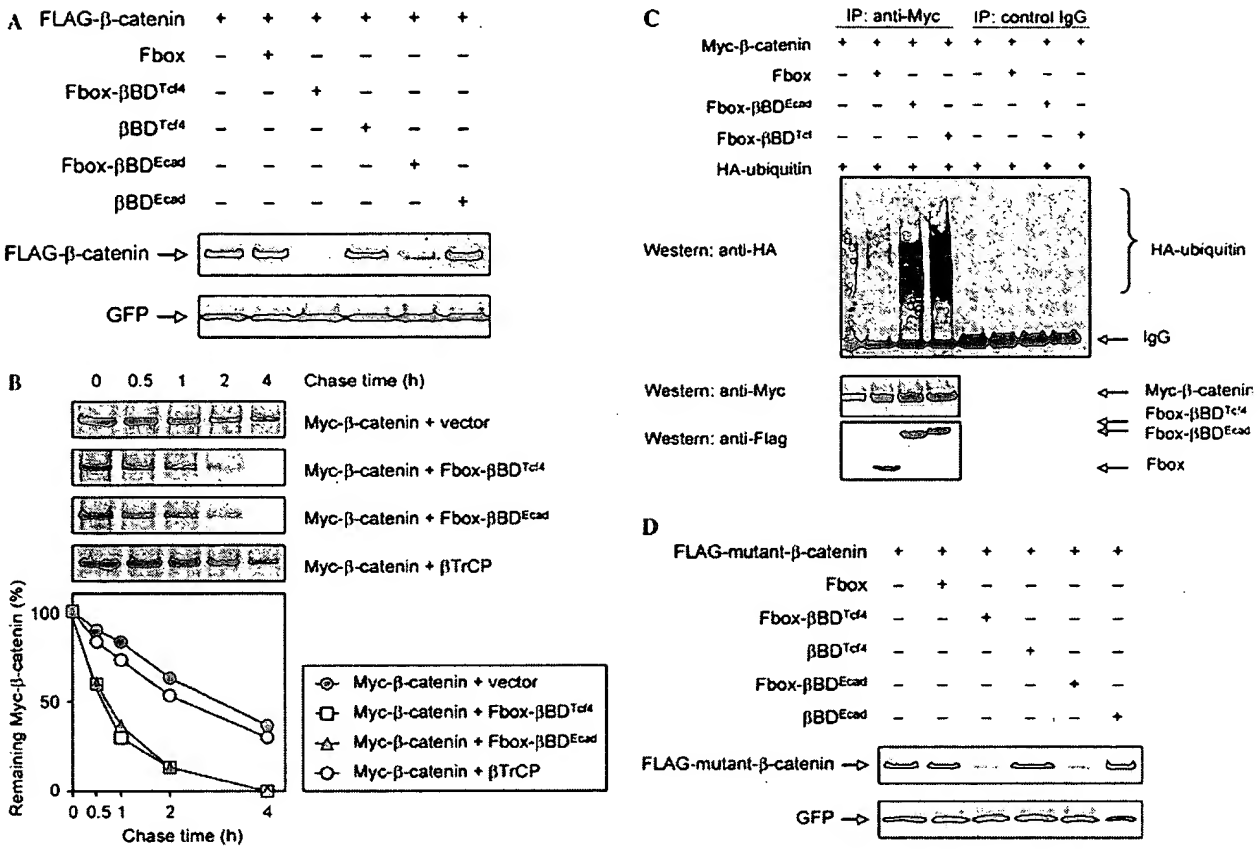

Fig. 3. Effect of chimeric F-box fusion proteins on β-catenin levels and turnover. (A) 293T cells were transiently co-transfected with an internal control (pEGFP) and FLAG-tagged β-catenin in a combination with indicated plasmids. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Whole cell lysates were subjected to Western analysis. Blots were probed with mouse monoclonal antibodies to FLAG epitope tag (upper panel) and GFP (lower panel). (B) Pulse-chase analysis of ectopically expressed Myc-tagged β-catenin. 239T cells were transiently co-transfected with the indicated plasmids, pulse-labeled with [^{35}S]methionine and -cysteine, and then chased with media lacking the labeled amino-acids. Cells were lysed at the indicated times and the expressed Myc-β-catenin was recovered by immunoprecipitation via a Myc epitope tag. Immunoprecipitated Myc-β-catenin was subjected to SDS–PAGE and dried gels were analyzed with a PhosphorImager. The data shown are a representative of multiple analyses. (C) Chimeric F-box fusion proteins promote ubiquitination of β-catenin. 293T cells were transfected with plasmids encoding Myc-tagged β-catenin and HA-tagged ubiquitin in combination with empty vector, Fbox, Fbox-βBD^{Ecad} or Fbox-βBD^{Tcf4} as indicated. Cells were treated with 10 μM MG132 for 12 h and the whole cell extract was immunoprecipitated with anti-Myc antibody, immunoblotted and probed with anti-HA antibody (upper panel). Five percent of each input lysate was immunoblotted and probed with anti-Myc antibody to show the expression level of Myc-tagged β-catenin (middle panel) or with anti-FLAG antibody to show the expression of indicated constructs (lower panel). The blots shown are representative of multiple experiments. (D) Chimeric F-box fusion proteins down-regulate β-catenin through a mechanism independent of GSK-3β-mediated phosphorylation. 293T cells were transiently co-transfected with an internal control (pEGFP) and FLAG-tagged mutant β-catenin in a combination with indicated plasmids. This mutant β-catenin carries phenylalanine and alanine substitutions of residues in putative GSK-3β phosphorylation sites (serine and threonine amino-acid residues between codons 33 and 45) [8]. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Whole cell lysates were subjected to Western analysis. Blots were probed with mouse monoclonal antibodies to FLAG epitope tag (upper panel) or GFP (lower panel). The blot shown is a representative of multiple experiments.

specific substrates by replacing the target recognition module.

Effect of chimeric F-box fusion proteins on mutant β-catenin

To determine whether Fbox-βBD^{Tcf4} and Fbox-βBD^{Ecad} regulate the abundance of β-catenin independently of a mechanism requiring GSK-3β-mediated phosphorylation, we examined the effect of Fbox-βBD^{Tcf4} and Fbox-βBD^{Ecad} on mutant β-catenin carrying phenylalanine and alanine substitutions of residues in putative GSK-3β phosphorylation sites (serine and threonine amino-acid residues between codons 33 and 45). Such substitutions produce β-catenin protein resistant to phosphorylation by GSK-3β and subsequent targeting for proteasome-mediated degradation, resulting in increased β-catenin stability [1,2]. Consistent with these observations, we have previously shown that overexpression of GSK-3β led to a decrease in the

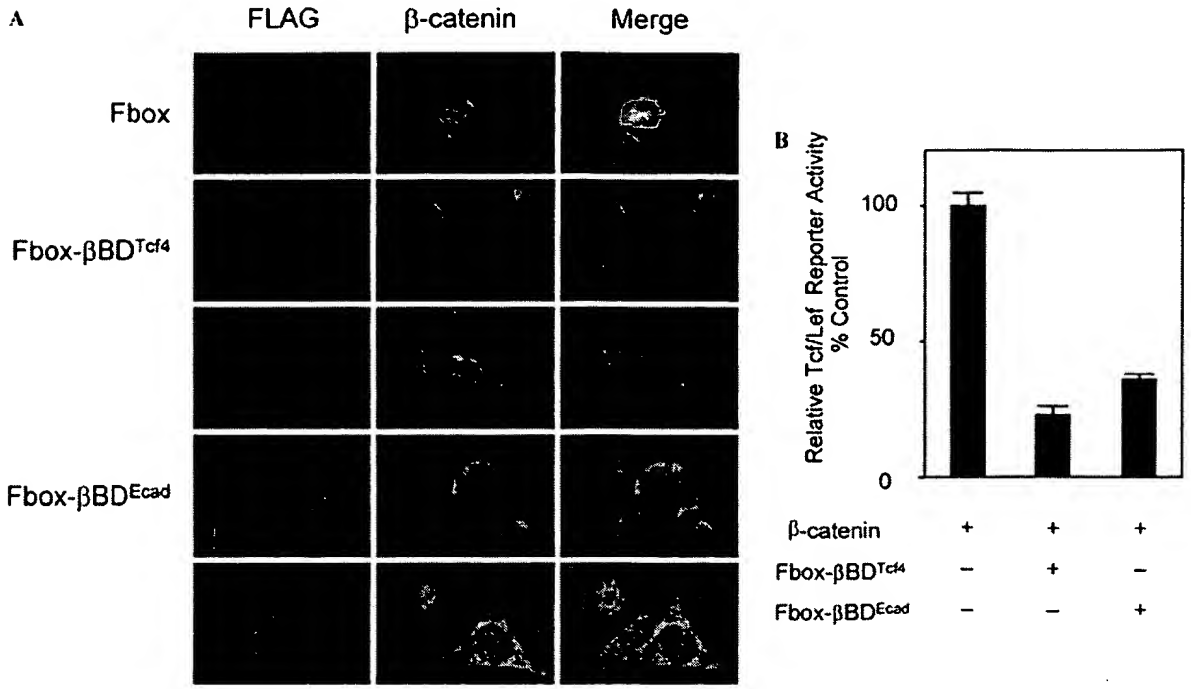

Fig. 4. Chimeric F-box fusion proteins down-regulate endogenous β-catenin in the absence of intact pAPC. (A) F-box fusion proteins down-regulate endogenous β-catenin in SW480 cells. Colon cancer cell line SW480 was transfected with indicated plasmids. Indirect immunofluorescence staining pictures indicate the expression of transfected Flag-tagged F-box constructs (Texas Red, red) and endogenous β-catenin (FITC, green). (B) F-box fusion proteins down-regulate β-catenin-mediated Tcf/Lef reporter activity in SW480 cells. SW480 cells were co-transfected with a reporter construct (pTOPFLASH or pFOPFLASH), an internal control (pCMVβ-gal), and indicated plasmids. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector. Luciferase and β-galactosidase activity was measured 24 h after transfection. Tcf/Lef reporter activity was determined as described [8] and the result is shown as relative Tcf/Lef reporter activity (TOP/FOP). The histograms are presented as the average ± SD from multiple experiments.

amount of wild-type FLAG-β-catenin but had no effect on the amount of mutant FLAG-β-catenin [8]. As shown in Fig. 3D, expression of Fbox-βBD^{Tcf4} or Fbox-βBD^{Ecad} led to a substantial decrease in the amount of mutant β-catenin. These results indicate that both Fbox-βBD^{Tcf4} and Fbox-βBD^{Ecad} mediate down-regulation of β-catenin independently of GSK-3β-mediated phosphorylation.

Effect of chimeric F-box fusion proteins on the amount of endogenous β-catenin in colon cancer line SW480 with APC mutations

Intact pAPC is required to facilitate β-catenin proteolysis through its endogenous degradation pathways [1,2,8,10]. APC mutations frequently occur in colon cancers, which lead to disruption of essential β-catenin-degradation pathways and subsequently result in accumulation of β-catenin. To test if F-box fusion proteins facilitate degradation of β-catenin in the absence of intact pAPC, we transiently introduced chimeric constructs into a colon cancer cell line SW480 with APC mutations and examined its effect on the amount of endogenous β-catenin by immuno-staining. SW480 shows truncated mutant pAPC and an elevated level of β-catenin [4]. As shown in Fig. 4A, SW480 cells transfected with Fbox-βBD^{Tcf4} and Fbox-βBD^{Ecad} showed dramatically decreased β-catenin staining. On the other hand, cells transfected with a construct expressing only the F-box domain had no effect. To test if F-box fusion proteins affect β-catenin-mediated transcriptional activities in the absence of intact pAPC, we transiently transfected SW480 cells with F-box fusion constructs. As shown in Fig. 4B, expression of both F-box fusion constructs robustly decreased the β-catenin-mediated Tcf/Lef reporter activities in the absence of intact pAPC. These results demonstrate that the cellular SCF machinery can be re-directed to degrade specific cellular proteins through an engineered F-box-containing fusion protein without facilitation of certain essential components, which are required for endogenous degradation pathways and frequently mutated in cancers, such as APC.

Discussion

The major β-catenin degradation mechanisms are largely known and have been extensively studied. It is desirable to engineer a system to specifically degrade

β-catenin and its mutant forms independent of pAPC based on the known mechanisms of protein degradation. In this study, we demonstrated that engineered chimeric F-box proteins specifically bind and target β-catenin for ubiquitin-mediated degradation. It is independent of both β-catenin phosphorylation status and an intact pAPC.

The SCF complex bridges the association between the E2 ubiquitin-conjugating enzymes and substrates to facilitate ubiquitin transfer [11]. Multiple earlier studies have been reported to explore this ubiquitin conjugation machinery as a tool for targeted proteolysis [12–14]. F-box proteins are critical components of the SCF complex. They are involved in substrate binding and recruitment for other components of the SCF complex. Engineered F-box like proteins could facilitate targeted proteolysis by binding substrates with specific protein interaction domains.

We created β-catenin-specific F-box fusion proteins by replacing the WD40-repeat of β-TrCP with β-catenin-binding domains (βBD) of Tcf4 and E-cadherin. These βBDs interact with the central armadillo repeat region of β-catenin [9] independent of its phosphorylation status. We have also shown that the F-box fusion proteins bind both β-catenin and Skp1, a key component of the SCF complex, thus bringing the target and the ubiquitin-conjugating machinery together. Expression of chimeric F-box proteins substantially increased the ubiquitination level of β-catenin, which supports the hypothesis that the physical interaction between a ubiquitin-conjugating machinery and its substrate might be sufficient for ubiquitination reactions to occur. Interestingly, the specific fusion configuration of the F-box chimera, with the F-box domain at the amino-terminus and the interaction module at the carboxyl terminus, is essential for its function (data not shown). This particular orientation is the same as those of most endogenous F-box proteins. It is also worth noting that both β-catenin interaction modules from E-cadherin and Tcf4 bind the central armadillo repeat region of β-catenin, an extended surface close to the N-terminal motif responsible for binding β-TrCP. Therefore, a specific spatial orientation of the chimeric F-box protein and its target, β-catenin, within the ubiquitination complex might be required for an efficient ubiquitination reaction.

In order to degrade β-catenin in cancer cells, it is necessary to design a β-catenin-eliminating molecule functioning in the absence of wild-type APC. APC mutations have been found in more than 75% of inherited and sporadic colorectal cancers, leading to disruption of two major β-catenin degradation pathways, one through GSK-3β and β-TrCP and the other through p53 and Siah-1 [2,8,10]. Unlike β-TrCP, the engineered F-box fusion proteins do not require intact pAPC to promote β-catenin down-regulation. When chimeric F-box proteins were expressed in SW480 colorectal cancer cells with APC truncating mutations, β-catenin was dramatically down-regulated, demonstrated by immnostaining using anti-β-catenin antibody. Furthermore, β-catenin/TCF-mediated transcription activity was robustly repressed, based on a reporter assay with the luciferase gene driven by the TCF-binding elements.

Another frequently reported somatic mutation in various types of cancers occurs at the amino-terminus of β-catenin that alters putative GSK-3β phosphorylation residues. Although the central region of APC or full length Axin could down-regulate wild-type β-catenin [4,5], neither of them is capable of down-regulating the mutant forms of β-catenin, due to lack of interaction between the mutant β-catenin and β-TrCP. Re-directing the ubiquitin-conjugating complex to β-catenin independent of its phosphorylation status provides a robust method to overcome the oncogenic, mutant forms of β-catenin.

The strategy described in this report provides an additional dimension to target pathogenic proteins and ultimately cure cancerous cells. Further work to identify specific interaction modules capable of distinguishing wild-type proteins and its mutant forms would make this strategy more valuable, especially in heterozygous samples.

While this manuscript was in preparation, two other groups have described similar approaches for β-catenin degradation [15,16]. The fusion constructs from the three groups are based on the similar principles, but not identical, which supports the notion that enforced proximity between selected targets and the ubiquitin-conjugation machinery might be the most important fact in facilitating specific proteolysis. Although both papers showed down-regulation of the abundance of β-catenin upon expression of the F-box chimera, it is not clear whether its functions through accelerated protein degradation. With pulse-chase experiments, we showed β-catenin's half-life was dramatically reduced upon expression of our F-box chimera, which demonstrates the chimera approach indeed facilitates specific proteolysis.

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Exploring the functional complexity of cellular proteins by protein knockout

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Comprehensive dissection of protein functions entails more complicated manipulations than simply eliminating the protein of interest. Established knockdown technologies, such as RNA interference, antisense oligodeoxynucleotides, or ribozymes, are limited for specific applications such as modulating protein levels or specific targeting of a posttranslationally modified subpopulation. Here we show that the engineered Skp1, Cullin 1, and F-box-containing βTrCP substrate receptor ubiquitin-proteolytic system, designated protein knockout, could achieve not only total elimination but also rapid and systematic reduction of a given cellular protein. Stable expression of a single engineered βTrCP demonstrated simultaneous and sustained degradation of the entire retinoblastoma family proteins. Furthermore, the engineered βTrCP was capable of selecting hypo- but not hyperphosphorylated forms of retinoblastoma for degradation. The engineered βTrCP has been extensively modified to increase its specificity in substrate selection. This optimized protein-knockout system offers a powerful and versatile proteomic tool to dissect diverse functional properties of cellular proteins in somatic cells.

Understanding the function of a given cellular protein often requires alterations of its steady-state levels to examine the associated physiological consequences. Overexpression of the gene of interest has been extensively used to assess its functions. However, this method may generate gain-of-function phenotypes instead of revealing the genuine physiological properties. A number of approaches that aim at removing a cellular protein in somatic cells have been developed over the years, which include antisense deoxyoligonucleotides, ribozyme, and triple-helix DNA. Recently, RNA interference (RNAi) has been extensively used to block the biosynthesis through mRNA destruction and thus reduces the levels of a given cellular protein. All these technologies function at various levels of biosynthesis and rely on the endogenous protein degradation apparatus to remove the existing target protein. As a result, functional assessment of knockdown or knockout phenotype relies largely on the intrinsic stability of individual target proteins. The turnover rate of a given cellular protein also determines the kinetic rate of a knockdown and thus affects the analysis of primary (or immediate) versus secondary (or delayed) responses associated with the down-regulation of a specific target protein. Protein knockout is an efficient alternative approach, which involves specific engineering of the cellular ubiquitination machinery to directly remove specific proteins through accelerated proteolysis (1).

The specificity of a given ubiquitin-proteolytic pathway is conferred exclusively by the E3 ubiquitin–protein ligase (reviewed in ref. 2). One of the extensively characterized E3s is the multimeric $SCF^{\beta TrCP}$ complex, consisting of Skp1, Cullin 1, F-box-containing substrate receptor, βTrCP, and the RING domain protein Rbx1/Roc1/Hrt1 (3–7). The substrate receptor, βTrCP, is a modular protein with two distinct domains; the F-box that binds to Skp1 to connect to the core SCF complex, and the WD40 domain at the C terminus, which is exclusively responsible for substrate recruitment. This modular configuration of the F-box-containing substrate receptor bears a striking resemblance to that of transcriptional factors, which carry structurally and functionally separable DNA binding and transcriptional activation (or repression) domains. Remarkably, these two distinct classes of proteins operate similarly through regulated target recruitment (8).

Based on the modular nature of the F-box proteins, protein knockout was designed that introduces a protein–protein interaction module to the F-box-containing substrate receptors, and, thus, directing non-SCF substrates to the SCF machinery for ubiquitination and destruction (1). Similar modifications of the E6AP ubiquitin–protein ligase were also shown to promote ubiquitination of heterologous proteins (9). In addition, small synthetic ligands, designated Protacs, were designed to bridge the interaction of target protein with native βTrCP for destruction (10). In comparison to RNAi and antisense oligodeoxynucleotides, protein knockout is more effective in achieving complete eradication of target proteins. However, the use of the original chimeric F-box-containing ubiquitin–protein ligases was limited in its specificity for substrate selection. Furthermore, the application potential of the protein-knockout system has not been extensively explored. Here, we report an extensively refined protein knockout system with unique capabilities to dissect detailed functional properties of cellular proteins of interest in somatic cells.

Materials and Methods

Cell Lines, Plasmids, and Antibodies. C33A, U2OS, 293, 293T, and HeLa cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The HL-60 promyelocytic leukemia cell line was a kind gift of Andrew Yen (Cornell University, Ithaca, NY) and was maintained in RPMI medium 1640 supplemented with 5% FCS. The pcDNA-F-TrCP, pcDNA-F-TrCP-E7N, and pcDNA-F-TrCP-E7N(ΔDLYC) plasmids have been described (1). FLAG-tagged βTrCP(m1)-E7N, which carries five amino acid substitutions (R285E, K365E, R474E, R521E, and R524E), was generated by using the QuikChange multisite-directed mutagenesis kit (Stratagene). F-TrCP-10GS-E7m, which carries the minimal retinoblastoma (RB)/p107/p130-binding domain derived from the E7 protein of the human papillomavirus type 16 (HPV16) and preceded by 10 copies of Gly-Ser (GS) repeats, was generated by PCR and cloned into pcDNA3 (Invitrogen). F-TrCP-E7N, F-TrCP(m1)-E7N and F-TrCP-E7N(ΔDLYC) were also cloned into the *Bam*HI/*Not*I sites of the pBMN-GFP retroviral plasmid (Orbigen, San Diego). pBMN-GFP is a bicistronic retroviral vector that also expresses GFP from the Moloney murine leukemia virus long terminal repeat (LTR) and the internal ribosomal entry site. Antibodies against RB, p107, p130, cyclin A, cdk2, and IκB were purchased from Santa Cruz Biotechnology, FLAG (M2) was from Sigma, and hypophosphorylated and hyperphosphorylated RB were from BD Biosciences and Cell Signaling Technology (Beverly, MA), respectively. Indirect immunofluorescence assay was performed as described (11).

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Abbreviations: ATRA, *all-trans* retinoic acid; RB, retinoblastoma; RNAi, RNA interference; SCF, Skp1, Cullin 1, and F-box-containing substrate receptor; FACS, fluorescence-activated cell sorter.

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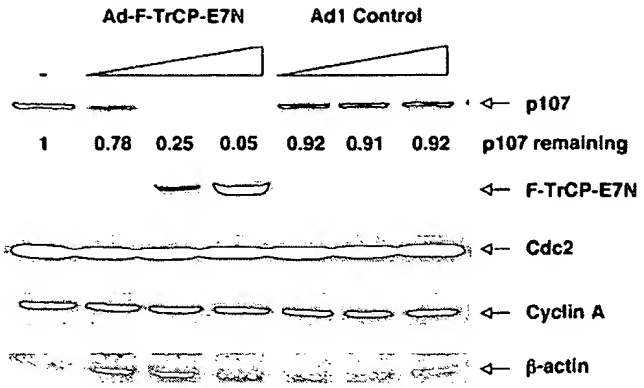

Fig. 1. Systematic reduction of the endogenous p107 by F-TrCP-E7N. C33A cells were infected with increasing doses of recombinant Ad-F-TrCP-E7N adenovirus for 48 h. The levels of endogenous p107, cyclin A, Cdk2, and β-actin (loading control) were detected by immunoblotting with the respective antibodies, and in comparison with the dose-dependent increase in the expression of F-TrCP-E7N. The percentages of p107 remaining in each adenovirus-infected cell were quantitated by densitometry scanning and are indicated.

Transfection and Retroviral Infection. Transient transfections of C33A cells were performed by calcium phosphate precipitation (Promega). One microgram of pCMV-CD19 was included in transient transfections, and transfected C33A cells were enriched by using the anti-CD19 immunomagnetic beads (1). To generate recombinant retroviruses, the pBMN-F-TrCP-E7N, pBMN-F-TrCP-E7N(ΔDLYC), or pBMN-GFP plasmids were transfected into 293T cells together with the SV-A-MLV-ENV and pEQ-PAM3(−E) plasmids carrying the amphotropic murine leukemia virus *env* gene and the *gag/pol* genes, respectively (12). The viral supernatant was harvested at 24, 48, and 72 h and was used for infection of the HL-60 cells to assess degradation of the RB family proteins. Fluorescence-activated cell sorter (FACS) analysis was carried out to isolate infected HL-60 cells, which was based on the expression of GFP from the recombinant retroviruses.

Results

Modulation of Cellular p107 Levels Through Controlled Proteolysis. We have previously shown that the endogenous p107, a member of the RB family of proteins, which is not a natural substrate of $SCF^{\beta TrCP}$, can be eliminated in the C33A cervical carcinoma cells by the ectopic expression of a chimeric βTrCP ubiquitin–protein ligase (βTrCP-E7N) that carries a p107-binding peptide derived from the N-terminal 35-aa residues of the HPV oncoprotein, E7 (E7N) (1). However, the functions of many cellular proteins are dictated by their intracellular abundance, and not simply by their presence or absence. Thus far, there has not been a reliable method to precisely modulate the intracellular protein levels in mammalian cells.

We first investigated whether p107 levels could be systematically reduced through controlled expression of different amounts of βTrCP-E7N. The human C33A cervical carcinoma cells were infected with increasing doses of recombinant adenovirus carrying βTrCP-E7N or the control adenovirus for 24 h. Infection was 100%, even at the lowest dose [a multiplicity of infection (moi) of 10] used, as judged by the expression of GFP carried by the adenovirus in all recipient cells (data not shown). As shown in Fig. 1 (*Upper*), the endogenous p107 levels were systematically reduced to 78%, 25%, and 5% of the steady-state levels when C33A cells were transduced with recombinant βTrCP-E7N adenovirus at mois of 10, 35, and 80, but not the same doses of Ad1 control virus. Therefore, the engineered $SCF^{\beta TrCP\text{-}BP}$ offers a simple and reproducible means to reduce the overall amounts of target proteins at defined levels for assaying the dosage effects on biological activities.

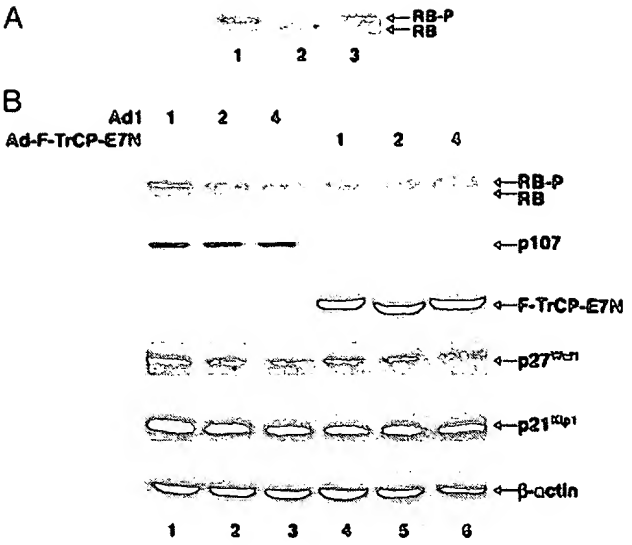

Fig. 2. Selective degradation of hypophosphorylated form of RB in U2OS cells. (*A*) U2OS cells contain both hypophosphorylated and hyperphosphorylated forms of RB. Immunoblotting was carried out to detect RB in U2OS cell extracts by using antibodies that recognize both RB forms (lane 1) (IF8, Santa Cruz Biotechnology), or specific for hypo- (lane 2) (G99–549, BD Biosciences) or hyperphosphorylated RB (lane 3) [(phospho-RB (Ser 807/811) polyclonal antibody, Cell Signaling]. (*B*) U2OS cells were infected with increasing doses (in μl) of Ad1-F-TrCP-E7N or control pAd1 adenoviruses for 48 h. Cell extracts were prepared and immunoblotted with antibodies against RB, p107, FLAG (M2), $p21^{waf1}$, $p27^{kip1}$, and β-actin.

P107 stably associates with cyclin A and Cdk2 through a high-affinity binding site in proliferating cells (13–15). To examine whether these p107-associated proteins are also subjected to targeted degradation by βTrCP-E7N, immunoblotting was performed in C33A cells infected by the Ad-F-TrCP-E7N virus. As shown in Fig. 1, the endogenous cyclin A and Cdk2 levels remained unaltered, whereas p107 was dramatically down-regulated. Furthermore, the levels of nontargeted β-actin were not affected by F-TrCP-E7N (Fig. 1). This result provided strong evidence that the engineered βTrCP-E7N ubiquitin–protein ligase specifically degrades targeted substrate p107, but not its associated proteins.

Selective Degradation of a Specific Posttranslationally Modified Subpopulation of Target Protein. Modification of a cellular protein at the posttranslational level, such as phosphorylation, is a fundamental means cells employ to regulate the function or to confer new activities of the protein. Experimental tools to dissect the function associated with a specific posttranslational modification event are currently limited. Because protein knockout operates at the posttranslational level that directly recognizes target proteins, one application is to engineer a specific E3 that is capable of selecting a subpopulation of posttranslationally modified proteins for degradation, instead of destroying the entire population.

The HPV16 E7 was found to selectively bind to the hypophosphorylated form of RB (16). We investigated whether the chimeric βTrCP-E7N ubiquitin–protein ligase could distinguish hypo- and hyperphosphorylated RB and select only the hypoform for degradation. The human U2OS osteosarcoma cells express both forms of RB that can be readily identified, based on their different electrophoretic mobility on SDS/PAGE (Fig. 2*A*, lane 1) (17). The identity of these two RB species was further verified by immunoblotting using antibodies specific for either hypo- or hyperphosphorylated forms of RB (Fig. 2*A*, lanes 2 and 3). U2OS cells were infected with the recombinant Ad-F-TrCP-E7N or the control Ad1 adenoviruses for 48 h, and the steady-state levels of RB-P and RB were analyzed

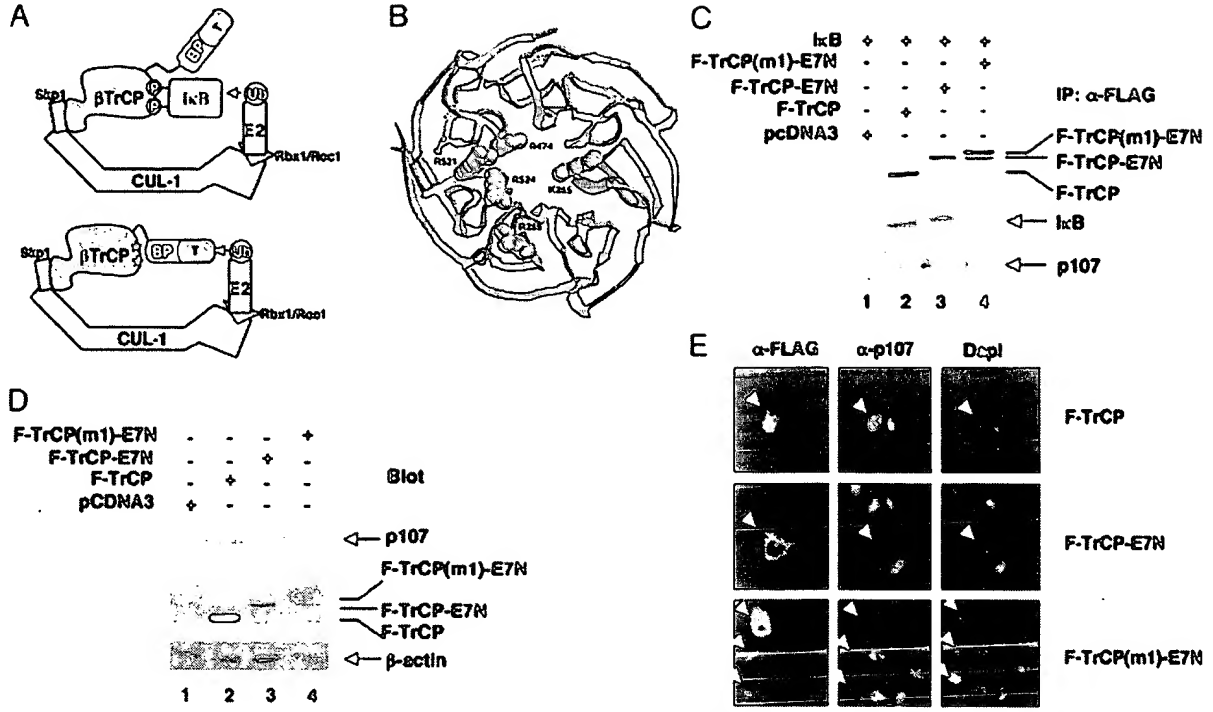

Fig. 3. Structure-based mutagenesis of the substrate-binding residues on the engineered βTrCP-E7N ubiquitin–protein ligase. (*A*) Schematic diagrams of the predicted $SCF^{\beta TrCP\text{-}BP}$/IκB/target and $SCF^{\beta TrCP(m1)\text{-}BP}$/target complexes. BP, binding peptide; T, target. (*B*) Three-dimensional model of the WD40 repeats of βTrCP. The five positively charged residues are cyan. (*C*) The modified F-TrCP(m1)-E7N ubiquitin–protein ligase cannot bind to IκB, but retains its interaction with p107. HeLa cells were transiently transfected with IκB, together with pcDNA3 (lane 1), F-TrCP (lane 2), F-TrCP-E7N (lane 3), and F-TrCP(m1)-E7N (lane 4), and treated with MG132 for 2 h and then with TNF-α for 15 min. Cell extracts were prepared for immunoprecipitation with the anti-FLAG (M2) antibody, and probed with either phosphorylated IκB antibody or anti-p107 polyclonal antibody. F-TrCP(m1)-E7N migrates as a doublet on SDS gels for reasons yet to be determined (lane 4). (*D*) Efficient degradation of p107 by F-TrCP(m1)-E7N in C33A cells. C33A cells transfected with the indicated plasmids and 1 μg of pCMV-CD19 were enriched by immunomagnetic selection, and levels of endogenous p107 were examined by immunoblotting. (*E*) Indirect immunofluorescence assay was carried out to detect endogenous p107 (*Center*) in response to transient transfection and expression of F-TrCP, F-TrCP-E7N, or F-TrCP(m1)-E7N (*Left*) in individual C33A cells (marked by arrows). Images of the same field of cells counterstained with 4′,6-diamidino-2-phenylindole (DAPI) were shown to identify the nuclei of both transfected (marked by arrows) and untransfected cells.

simultaneously by Western blotting, using an anti-RB monoclonal antibody that recognizes both forms. Consistent with the preferential binding of E7N to hypophosphorylated RB, ectopic expression of F-TrCP-E7N resulted in the elimination of hypophosphorylated RB, whereas the hyperphosphorylated RB-P was left intact (Fig. 2*B Upper*, compare lanes 4–6 with lanes 1–3). This result highlights the capability of the engineered F-TrCP-E7N ubiquitin–protein ligase in selecting a specific subpopulation of RB for degradation, which was based on the status of certain posttranslational modification of the target protein.

Full-length HPV16 E7 could interact with the cyclin-dependent kinase inhibitors $p21^{Waf1}$ and $p27^{Kip1}$, but the binding domain was mapped to the C-terminal domain (residues 40–98), instead of the E7N fragment (residues 18–20). To further examine the specificity of F-TrCP-E7N, the same U2OS cell extracts were also probed with antibodies against $p21^{Waf1}$ and $p27^{Kip1}$. As seen in Fig. 2*B* (*Middle*), the steady-state levels of $p21^{Waf1}$ and $p27^{Kip1}$ remained unaltered. Taken together, the engineered F-TrCP-E7N demonstrated proteolytic specificity toward the RB family proteins, but not other cell-cycle regulators such as Cdk2, cyclin A, $p21^{Waf1}$, and $p27^{Kip1}$.

Structure-Based Mutagenesis to Generate a Highly Specific βTrCP-E7N Ubiquitin–Protein Ligase. The chimeric βTrCP-E7N is still capable of binding the endogenous βTrCP substrates, including IκB, β-catenin, and ATF4 (3–7). Overexpression of βTrCP-E7N might interfere with degradation of these native substrates. Conversely, binding of the endogenous substrate to βTrCP-E7N might interfere with the proper positioning of the intended substrate to accept ubiquitin from ubiquitin-conjugating enzyme, and therefore reduce the efficiency of targeted degradation (Fig. 3*A Upper*). Furthermore, βTrCP interacts with a pseudo substrate hnRNP-U, which tethers βTrCP and its derivatives in the nucleus and precludes their interaction with the substrates (21). By introducing specific mutations of the amino acid residues on βTrCP to eliminate its binding to β-catenin, IκB, or hnRNP-U, we anticipate to improve not only the specificity but also the availability of the engineered βTrCP-E7N to recruit the intended substrate (Fig. 3*A Lower*).

The substrate-binding domain of βTrCP contains seven WD40 repeats in the C-terminal region, which fold into the typical seven-bladed β-propeller structure conserved among WD40 proteins (3–7). Because the overall integrity of the β-propeller structure of WD40 proteins is critical for their functions, standard deletion mutagenesis approach will likely induce gross structural alterations, and, thus, is not applicable for defining substrate-binding sites on βTrCP. However, the known crystal structure of WD40 proteins can be exploited to identify surface residues involved in binding βTrCP substrates. A structure-based mutagenesis approach was designed to identify and mutate five positively charged residues (R285, K365, R474, R521, and R524), predicted to reside on the top surface of the WD40 β-propeller, which are involved in binding to WD40-associated proteins (Fig. 3*B*) (22). This approach was detailed in *Supporting Methods*, which is published as supporting information on the PNAS web site. Also, see Fig. 6, which is published as supporting information on the PNAS web site. Furthermore, substitutions of these Lys/Arg residues will not likely alter the overall βTrCP structure.

By using the QuikChange multisite-directed mutagenesis kit

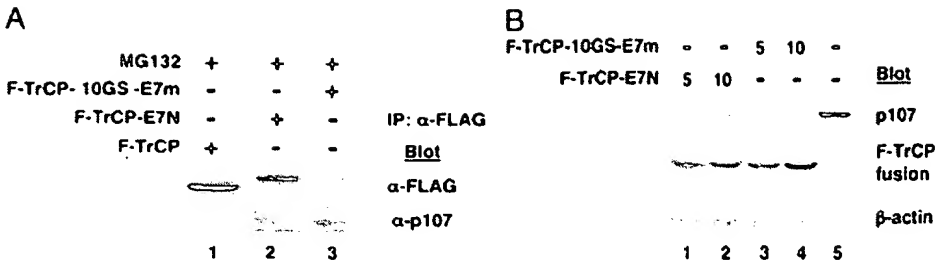

Fig. 4. The minimal p107-binding domain of E7 is sufficient to recruit p107 for targeted destruction. (*A*) The engineered F-TrCP-10GS-E7m ubiquitin–protein ligase efficiently binds to p107. C33A cells transiently transfected with the indicated plasmids were treated with the proteasome inhibitor MG132 for 2 h. Cell extracts were prepared for immunoprecipitation with the anti-FLAG antibody, and were probed with antibodies against FLAG or p107. (*B*) Degradation of the endogenous p107 by the engineered F-TrCP-E7m. C33A cells were transiently transfected with the indicated plasmids (in μg) and 1 μg of pCMV-CD19. Transfected cells were enriched by immunomagnetic bead selection, and the steady-state levels of p107, F-TrCP fusions, and β-actin (loading control) were determined by immunoblotting using antibodies against p107, FLAG, and β-actin. The experiment was repeated four times, and the amount of p107 remaining was measured by PhosphorImager analysis. Endogenous β-actin levels were also measured as an internal loading control.

(Stratagene), we have simultaneously mutated these residues to Glu. This FLAG-tagged compound mutant, designated F-TrCP(m1)-E7N, was tested for its binding to both the endogenous (IκB) and the intended (p107) knockout targets. HeLa cells were transiently transfected with F-TrCP(m1)-E7N or F-TrCP-E7N, and their interactions with IκB and p107 were determined by coimmunoprecipitation. F-TrCP-E7N and F-TrCP exhibited similar affinities to the phosphorylated IκB protein, whereas the compound m1 mutations abolished F-TrCP(m1)-E7N binding (Fig. 3*C Middle*). In contrast, similar levels of p107 were detected in the immunocomplexes of F-TrCP-E7N or F-TrCP(m1)-E7N (Fig. 3*C Lower*), indicating that the engineered F-TrCP(m1)-E7N ubiquitin–protein ligase is fully functional in recruiting the intended cellular targets. These results are in agreement with the structural predictions in Fig. 3*B*, and further define the amino acid residues on βTrCP critical for endogenous substrate recognition.

The engineered F-TrCP(m1)-E7N was further analyzed for its potency in degrading endogenous p107. C33A cells were cotransfected with F-TrCP, F-TrCP-E7N, or F-TrCP(m1)-E7N, together with a CD19 expression plasmid. Forty-eight hours after transfection, cells were harvested and enriched for those receiving the transfected plasmids by using the immunomagnetic beads conjugated to the anti-CD19 monoclonal antibody. Endogenous p107 levels were subsequently determined by immunoblotting. As shown in Fig. 3*D*, ectopic expression of F-TrCP(m1)-E7N resulted in a 95% reduction of endogenous p107 levels, whereas an 82% down-regulation of p107 was observed for F-TrCP-E7N (Fig. 3*D Upper*, compare lanes 4 and 3). Furthermore, expression of F-TrCP(m1)-E7N had no observable effect on tumor necrosis factor (TNF)-α induced degradation of IκB by the native $SCF^{\beta TrCP}$, or on destruction of $p27^{kip1}$ by the SCF^{Skp2} ubiquitin–protein ligases (data not shown). Therefore, ectopic expression of the engineered F-TrCP(m1)-E7N does not interfere with the overall SCF ubiquitination activity by squelching the core SCF components (Skp1, CUL-1, and Rbx1/Roc1) shared by the endogenous F-box proteins.

Targeted p107 degradation was further assessed in individual C33A cells by indirect immunofluorescence assay. Consistently, p107 was largely eradicated in C33A cells expressing F-TrCP-E7N or F-TrCP(m1)-E7N, but not F-TrCP alone (Fig. 3*E*). Collectively, these results indicate that by eliminating the binding sites of the endogenous βTrCP substrates, the engineered F-TrCP(m1)-E7N ubiquitin–protein ligase demonstrated increased specificity and robust proteolytic activity toward the intended target.

Minimal Binding Domain as Targeting Peptide for Efficient Substrate Recruitment. To achieve high specificity of substrate recruitment and to minimize nonspecific targeting of other cellular proteins, we tested whether the minimal p107/RB interaction domain of E7N (designated E7m), which encompasses residues 21–29 of E7 spanning the LXCXE motif (23), could achieve efficient binding and degradation of p107. A chimeric ubiquitin–protein ligase was constructed in which βTrCP and E7m were covalently linked together by a flexible 20-aa spacer comprised of 10 copies of Gly-Ser repeats (designated 10GS). The F-TrCP-E7N or F-TrCP-10GS-E7m were transiently transfected into C33A cells, and their binding to the endogenous p107 was assessed by immunoprecipitation in extracts prepared after treatment with the proteasome inhibitor MG132 to inhibit degradation as a consequence of this interaction. As shown in Fig. 4*A* (lanes 2 and 3), F-TrCP-10GS-E7m had slightly increased affinity to p107 than that of the original F-TrCP-E7N. The F-TrCP-E7N and F-TrCP-10GS-E7m were further evaluated for their efficiency in p107 degradation by transient transfection in C33A cells as in Fig. 3*D*. As shown in Fig. 4*B*, F-TrCP-E7N and F-TrCP-10GS-E7m induced up to 92% and 95% down-regulation of p107, indicating that the engineered βTrCP carrying the minimal p107-binding domain functions as efficiently as the original F-TrCP-E7N in degrading target proteins.

Retroviral-Mediated Delivery of the Engineered βTrCP for Sustained Degradation of Endogenous Targets. We next examined whether the engineered ubiquitin–protein ligases could be stably expressed to achieve sustained degradation of the intended target. The HL-60 promyelocytic leukemia cells were transduced with the recombinant retroviruses expressing PBMN-GFP, F-TrCP(m1)-E7N, or the control F-TrCP-E7N(ΔDLYC), in which the RB/p107-binding site was deleted (1). Infected cells with chromosomally integrated chimeric F-TrCP transgenes were isolated by FACS sorting, based on the expression of GFP from the viruses, and degradation of the endogenous p107 was assessed from infected HL-60 cells either immediately after infection (Fig. 5*A*), or after 3 months of culturing in growth medium (Fig. 5*B*). Furthermore, because two other members of the RB family proteins, RB and p130, are also expressed in HL-60 cells, we examined whether a single F-TrCP-E7N can simultaneously target degradation of the entire RB family of proteins that interact with the same LXCXE motif of E7N. Ectopic expression of retrovirally transduced F-TrCP-E7N induced dramatic down-regulation of the hypophosphorylated form of RB, as well as p107 (Fig. 5*A*) and p130 (data not shown) in HL-60 cells, either immediately after infection and FACS sorting (Fig. 5*A*), or 3 months after infection and continuous culturing (Fig. 5*B*, lane 3). In contrast, stable expression of F-TrCP-E7N(ΔDLYC) in HL-60 cells had no effect on altering p107, RB (Fig. 5*A*, lane 3), and p130 levels (data not shown). Consistent with the observation that F-TrCP-E7N preferentially degrades hypophosphorylated RB in U2OS cells (Fig. 2), reduction of hyperphosphorylated RB species by stable expression of F-TrCP-E7N was less pronounced, compared with that of the hypophosphorylated form in HL-60 cells (Fig. 5*A*, compare lane 2 of pRB and pRB-P).

On treatment with *all-trans* retinoic acid (ATRA), HL-60 cells exit cell cycle and undergo terminal differentiation. An accumulation of hypophosphorylated RB was observed, which was previously reported to contribute to ATRA-induced growth arrest, whereas the hyperphosphorylated RB was not detectable (24, 25).

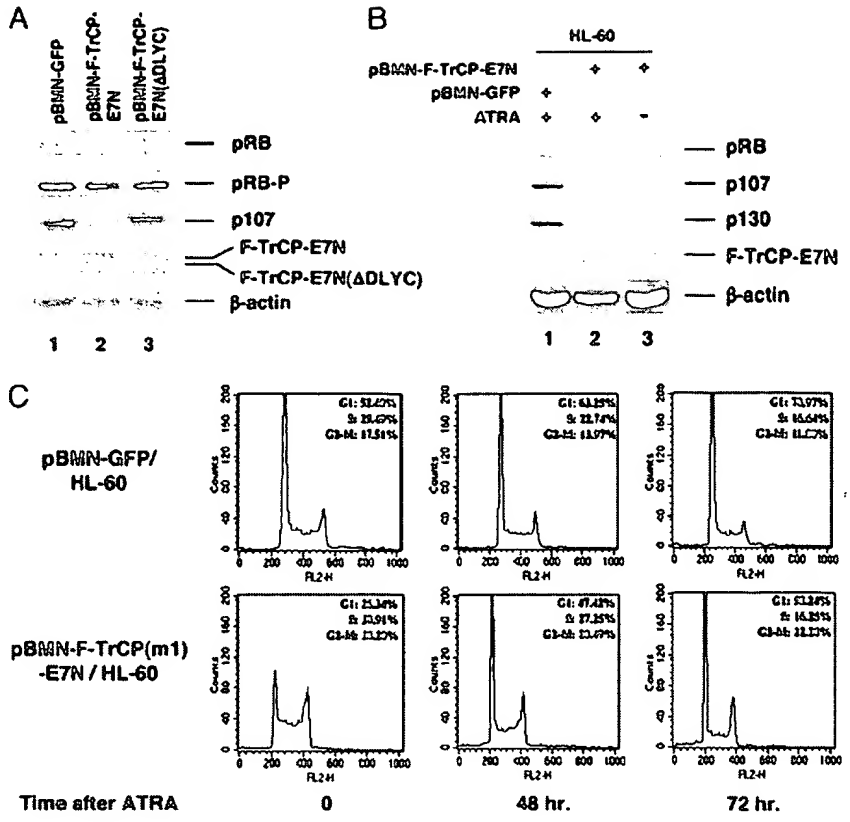

Fig. 5. Retroviral-mediated delivery of the engineered F-TrCP-E7N ubiquitin–protein ligase for targeted degradation of RB family proteins in the HL-60 cells. (*A*) HL-60 cells infected with the indicated recombinant retroviruses were isolated by FACS, and the steady state levels of RB, p107, F-TrCP-E7N, and F-TrCP-E7N(ΔDLYC) were determined by immunoblotting using the respective antibodies. β-Actin levels were also determined as a specificity and internal loading control. RB and RB-P denote hypo- and hyperphosphorylated RB. (*B*) Infected and FACS-sorted HL-60 cells were cultured for 3 months, and either untreated (lane 3) or treated with 1 μM of ATRA for 72 h (lane 2). Endogenous RB, p107, and p130 were determined in response to the expression of F-TrCP-E7N by immunoblotting. (*C*) FACS analysis to determine the DNA contents of the live HL-60 cells infected by control or pBMN-F-TrCP(m1)-E7N retroviruses under normal growth conditions and in response to ATRA-induced growth arrest.

To examine whether the $SCF^{\beta TrCP}$ machinery can also be harnessed during cell-cycle exit, HL-60 cells stably expressing F-TrCP-E7N or F-TrCP(m1)-E7N were treated with 1 μM ATRA for 72 h, and degradation of the RB family proteins were evaluated. HL-60 cells stably expressing F-TrCP-E7N resulted in 95%, 98%, and 85% decrease of RB, p107, and p130 levels respectively, compared with cells infected with the control pBMN-GFP virus (Fig. 5*B*, lane 2). Because ATRA also activates transcription of genes under the control of Moloney murine leukemia virus LTR of pBMN-GFP (26), the increased expression of F-TrCP-E7N on ATRA treatment likely further contributed to the efficient down-regulation of p107, p130, and hypophosphorylated RB (Fig. 5*B*).

The effect of the RB family proteins on normal cell-cycle progression has been examined in mouse embryo fibroblasts with disruptions in *RB*, *p107*, and *p130* genes, and found to be unresponsive to signals that induce G_1 arrest, such as growth factor withdraw or DNA damage (27, 28). However, the collective effects of the RB family members in tumor cell proliferation have not been studied, due to the lack of efficient reverse genetic tools. The stable HL-60 cells expressing F-TrCP(m1)-E7N from this study allowed us to assess how tumor cells deficient for all three RB family proteins responded to G_1-arrest signals. In exponentially growing HL-60 cells expressing F-TrCP(m1)-E7N that induced constitutive degradation of the RB family proteins, a marked acceleration of cell-cycle progression was observed in comparison with those infected by the control pBMN-GFP virus (Fig. 5*C Left*). This result is consistent with the impaired control of G_1–S transitions observed in $RB^{-/-}$, $p107^{-/-}$, and $p130^{-/-}$ triple knockout mouse embryo fibroblasts (27, 28).

ATRA treatment inhibited G_1-to-S-phase transition in HL-60 cells infected by the control pBMN-GFP (Fig. 5*C*) or F-TrCP-E7N(ΔDLYC) virus (data not shown), which is consistent with what was reported for uninfected HL-60 cells (Fig. 5*C*) (24). An initial delay in G_1–S transition was also observed in pBMN-F-TrCP(m1)-E7N/HL-60 cells at 48 h after ATRA. However, a complete G_1 arrest could not be achieved at the later stage (72–96 h) and cells continued the course of cell-cycle progression (Fig. 5*C*). In contrast, the control pBMN-GFP/HL-60 cells were all blocked in G_1 between 72 and 96 h. These observations suggest that F-TrCP(m1)-E7N-mediated degradation of RB family members impairs a late ATRA-response event necessary for completion of growth arrest, whereas the early attenuation of G_1–S progression was largely unaffected. Furthermore, a dramatic reduction in cell numbers was observed between 72 and 96 h post-ATRA treatment due to massive cell death during ATRA-induced cell-cycle exit and differentiation (29). Strikingly, HL-60 cells expressing F-TrCP(m1)-E7N exhibited an average increase of 2- to 3-fold in the $subG_1$ populations compared with that of the control virus infected HL-60 cells (data not shown), which is consistent with the increased cell death in $RB^{-/-}$, $p107^{-/-}$, and $p130^{-/-}$ triple knockout mouse embryonic fibroblast cells under growth-inhibitory conditions (28). Collectively, constitutive expression of F-TrCP(m1)-E7N induced down-regulation of the entire RB family proteins, which leads to an inhibition of growth arrest and an increase in cell death on ATRA treatment.

ATRA-induced G_1 arrest involves the coordinated regulation of multiple cellular signals/components that negatively control the cell cycle (reviewed in ref. 30). Dimberg *et al.* (31) reported that ATRA triggers a sequence of events in myeloid cells involving an early increase in $p21^{waf1}$ expression, stabilization of $p27^{kip1}$, and, later, dephosphorylation of RB at the onset of G_1 arrest. Because βTrCP(m1)-E7N only degrades RB family members and has no effect on the stability of $p21^{waf1}$ and $p27^{kip1}$, it is likely that only the late, RB-dependent ATRA response is affected, which is consistent with the resistance of pBMN-F-TrCP(m1)-E7N/HL-60 cells to complete G_1 arrest at 72–96 h (Fig. 5*C*). The observed early growth inhibition by ATRA at 48 h (Fig. 5*C*) could be attributed, at least

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in part, to the up-regulation of $p21^{waf1}$ and $p27^{kip1}$, whose stability is not affected by constitutive F-TrCP(m1)-E7N expression (Figs. 2 and 5).

Discussion

The engineered proteolysis system demonstrated rapid elimination, as well as systematic reduction of endogenous cellular proteins (such as p107). This latter property is due to the fact that the engineered βTrCP ubiquitin–protein ligases are likely functioning in a stoichiometric, but not catalytic manner. It is possible to control the engineered proteolytic activity by precisely modulating the expression levels of the engineered βTrCP, and this can be achieved through infection with different doses of recombinant adenoviruses. This fine-tuning property of the protein-knockout system offers a simple and reproducible means to study gene dosage effects in greater detail than a simple knockout or knockdown approach, and is especially valuable for studying essential proteins whose elimination is detrimental to cell growth or survival. Furthermore, the rapid kinetic rate of targeted proteolysis allows for functional evaluation as early as 8–12 h (see Fig. 7, which is published as supporting information on the PNAS web site), similar or even faster than what can be achieved by an inducible gene expression system. Thus, the protein-knockout approach allows rapid and controlled down-regulation of cellular proteins, which opens up avenues for detailed functional assessment of cellular proteins.

A second feature of the protein-knockout system is its ability to identify a specific subpopulation of cellular protein for destruction (Figs. 2 and 5*A*). Remarkably, the endogenous βTrCP itself has evolved the similar capability to recruit only the substrates that are Ser/Thr-phosphorylated for destruction, while leaving the unphosphorylated subpopulation intact (4). Because the protein knockout system operates at the posttranslational level, it offers a tool to delineate the functional consequences associated with specific posttranslational modifications. In this regard, other knockout or knockdown technologies that function through blocking protein synthesis, such as RNAi or antisense oligodeoxynucleotides, cannot make such distinctions.

The versatility of the protein knockout system is further highlighted by the observation that a single chimeric ubiquitin–protein ligase could simultaneously target degradation of multiple members of a protein family (Fig. 5). This feature offers a simple method to study a specific biological activity conferred by multiple protein family members that share redundant functions. In the event that individual family members need to be singled out to evaluate its contributions to a specific biological activity, targeting peptides against unique regions of the individual substrates should be used. Alternatively, single-chain antibody Fv fragment against a specific family member can be used as binding peptides with exquisite substrate specificity.

To facilitate the application of the protein-knockout system, several improvements have been made to increase the specificity of the engineered ubiquitin–protein ligases. They include (*i*) elimination of the binding sites for the endogenous βTrCP substrates; (*ii*) the use of minimal-binding domain as the targeting peptide; and (*iii*) stable expression of the engineered βTrCP for sustained degradation of the target of interest by using retroviral based vectors. Furthermore, the engineered βTrCP-E7N was shown to specifically target degradation of p107, but not cellular proteins that associate with p107, such as cyclin A and Cdk2. Based on the crystal structure of the $SCF^{\beta TrCP}$ complex (32), it is conceivable that p107 is likely positioned correctly between βTrCP-E7N and the ubiquitin-conjugating enzyme to accept ubiquitin transfer, whereas cyclin A and Cdk2 are either improperly positioned or misoriented, and, thus, incapable of receiving ubiquitin.

General applications of the protein-knockout system depend on the availability of specific targeting peptides for recruiting the desired substrate. The genome-wide screening of protein–protein interactions by the yeast two-hybrid assays is anticipated to add tremendous information to the current efforts to delineate protein complexes in the proteome, and further provide candidate targeting peptides for the protein-knockout vectors. Alternatively, targeting peptides can be obtained from screening the phage display or random peptide aptamer-based yeast two-hybrid libraries. Combined with RNAi, antisense, ribozyme, and other technologies, it is possible to achieve a comprehensive analysis of the functions of specific cellular proteins in somatic cells and in animals.

Note Added in Proof. While this paper was under revision, Wu *et al.* (32) published the crystal structure of the βTrCP-phosphorylated β-catenin complex. Remarkably, R285, R474, and R521, which we mutated in βTrCP(m1), were shown to be in direct contrast with β-catenin.

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A protein knockdown strategy to study the function of β-catenin in tumorigenesis

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Abstract

Background: The Wnt signaling pathway plays critical roles in cell proliferation and cell fate determination at many stages of development. A critical downstream target of Wnt signaling is the cytosolic β-catenin, which is stabilized upon Wnt activation and promotes transcription of a variety of target genes including c-myc and cyclin D. Aberrant Wnt signaling, which results from mutations of either β-catenin or adenomatous polyposis coli (APC), renders β-catenin resistant to degradation, and has been associated with multiple types of human cancers.

Results: A protein knockdown strategy was designed to reduce the cytosolic β-catenin levels through accelerating its turnover rate. By engineering a chimeric protein with the β-catenin binding domain of E-cadherin fused to βTrCP ubiquitin-protein ligase, the stable β-catenin mutant was recruited to the cellular SCF (Skp1, Cullin 1, and F-box-containing substrate receptor) ubiquitination machinery for ubiquitination and degradation. The DLD1 colon cancer cells express wild type β-catenin at abnormally high levels due to loss of APC. Remarkably, conditional expression of βTrCP-E-cadherin under the control of a tetracycline-repressive promoter in DLD1 cells selectively knocked down the cytosolic, but not membrane-associated subpopulation of β-catenin. As a result, DLD1 cells were impaired in their growth and clonogenic ability *in vitro*, and lost their tumorigenic potential in nude mice.

Conclusion: We have designed a novel approach to induce degradation of stabilized/mutated β-catenin. Our results suggest that a high concentration of cytoplasmic β-catenin is critical for the growth of colorectal tumor cells. The protein knockdown strategy can be utilized not only as a novel method to dissect the role of oncoproteins in tumorigenesis, but also as a unique tool to delineate the function of a subpopulation of proteins localized to a specific subcellular compartment.

Background

Wnt signaling plays diverse roles at many stages of development by regulating the stability of β-catenin [1]. In cells that do not receive a Wnt signal, cytoplasmic β-catenin is bound to a multi-protein β-catenin destruction complex that contains several proteins including Axin, APC, and

glycogen synthase kinase-3 (GSK3), and it is constitutively phosphorylated at a cluster of Ser and Thr residues at its N-terminus by GSK3. Phosphorylated β-catenin is recognized by βTrCP, a component of the $SCF^{\beta TrCP}$ ubiquitin-protein ligase complex, and degraded by the ubiquitin-proteasome pathway. Wnt signaling disassembles the β-catenin destruction complex, which prevents the phosphorylation and subsequent ubiquitination of β-catenin, thus diverting β-catenin from the proteasome machinery. Accumulated β-catenin then enters the nucleus, binds to the LEF/TCF family transcription factors, and activates the expression of β-catenin target genes.

Deregulated Wnt signaling contributes to tumorigenesis. Wnt-1, the founding member of the Wnt family, was first identified as a gene activated by insertion of a mouse mammary tumor provirus, leading to the formation of mouse mammary tumors [2]. Aberrant activation of Wnt signaling, which results from activating mutations of β-catenin or inactivating mutations of APC or Axin, has been associated with a wide variety of human malignancies, such as colorectal, heptocellular, ovarian endometrial, desmoid, and pancreatic tumors [3]. Among these tumor types, Wnt signaling is most frequently deregulated in colorectal tumors. APC is mutated in the majority of colorectal cancers, and those tumors with wild-type APC often contain mutated β-catenin [4]. Thus, aberrant activation of Wnt signaling appears to be obligatory for the initiation or progression of colorectal tumors. Recent studies suggested that β-catenin promotes tumorigenesis through increasing the expression of oncogenes like *c-myc* and *cyclin D1* [5–7].

β-catenin is a "dual function" protein, which is determined by its membrane and nuclear localizations. Membrane-associated β-catenin plays an important role in cell-cell adhesion. It binds to the intracellular domain of E-cadherin, and links E-cadherin to α-catenin and thereby to the cortical actin cytoskeleton. E-cadherin-mediated cell adhesion plays an inhibitory role in tumor invasion [8], and loss of E-cadherin promotes tumor progression [9]. Nuclear β-catenin enhances transcription of Wnt-responsive genes through interacting with TCF/LEF transcription factors and recruiting different transcriptional co-activators to the TCF/LEF binding sites.

To study the function of β-catenin in tumorigenesis, one needs to develop a strategy to selectively block the nuclear activity of β-catenin while leaving the membrane activity of β-catenin intact. Such an approach would enhance our understanding of the oncogenic function of β-catenin, and might further serve as a strategy for targeted therapy for tumors derived from aberrant Wnt signaling. In this study, a protein knockdown method was designed to induce the degradation of unphosphorylated β-catenin, which resulted in the suppression of neoplastic growth of colorectal tumor cells.

Results

Ubiquitin-dependent proteolysis constitutes the major pathway for eukaryotic cells to degrade specific proteins. This pathway involves a cascade of enzymatic reactions catalyzed by the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligase [10]. The substrate specificity of this system is determined by the E3 ligase. One such E3 ligase, designated SCF (Skp1, Cul-1, F-box-containing substrate receptor, and the Ring domain protein Rbx1/Roc1/Hrt1), is a multimeric protein complex that targets key regulators of cell cycle and signaling pathways for ubiquitination (reviewed in [11]). Among the SCF subunits, the F box proteins serve as receptors that recruit substrates through various protein-protein interaction domains and bring them to the core E3 (Skp1/Cul-1/Rbx1) through interaction between the F box and Skp1. βTrCP is such an F box protein that binds to its substrates, such as IκB and β-catenin, through its WD40 repeats. Specifically, serine phosphorylation of IκB and β-catenin is a prerequisite for their binding to βTrCP [12–15].

The SCF ubiquitination machinery can be harnessed to degrade a specific target protein by fusing an F box protein with a peptide that is able to bind to the target protein [16]. Here we investigated whether an F-box protein can be redesigned to target their usual substrates that have become resistant to degradation (eg, by mutations in the N-terminus of β-catenin). Recognition of β-catenin by βTrCP normally requires phosphorylation of serine residues within the N-terminal DSGxxS motif of β-catein [13,15]. To target unphosphorylated and thus stabilized β-catenin to the core SCF for ubiquitination and degradation, we fused the β-catenin binding domain of E-cadherin (amino acids 794–883, designated Ecad) to the C-terminus of βTrCP (Fig. 1A). A glycine-serine-rich sequence was inserted between βTrCP and Ecad to relieve the potential steric hindrance between these two protein structures. Ser37 is one major GSK3 phosphorylation site of β-catenin that is recognized by βTrCP [13]. Substitution of Ser37 with Ala abrogates the association between β-catenin and βTrCP. It has been shown that β-catenin S37A is about 9-fold more stable than the wild-type β-catenin [17]. The binding between β-catenin S37A and βTrCP-Ecad was assayed in 293 cells using a co-immunoprecipitation assay. HA-tagged β-catenin S37A and FLAG-tagged βTrCP-Ecad were coexpressed in 293 cells. Total cell lysates were immunoprecipitated with the anti-FLAG antibody, and immunoprecipitates were subjected to SDS PAGE and immunoblotting with the anti-HA antibody. As shown in Fig. 1B, β-catenin S37A strongly interacted with F-TrCP-Ecad, but not F-TrCP, indicating that the

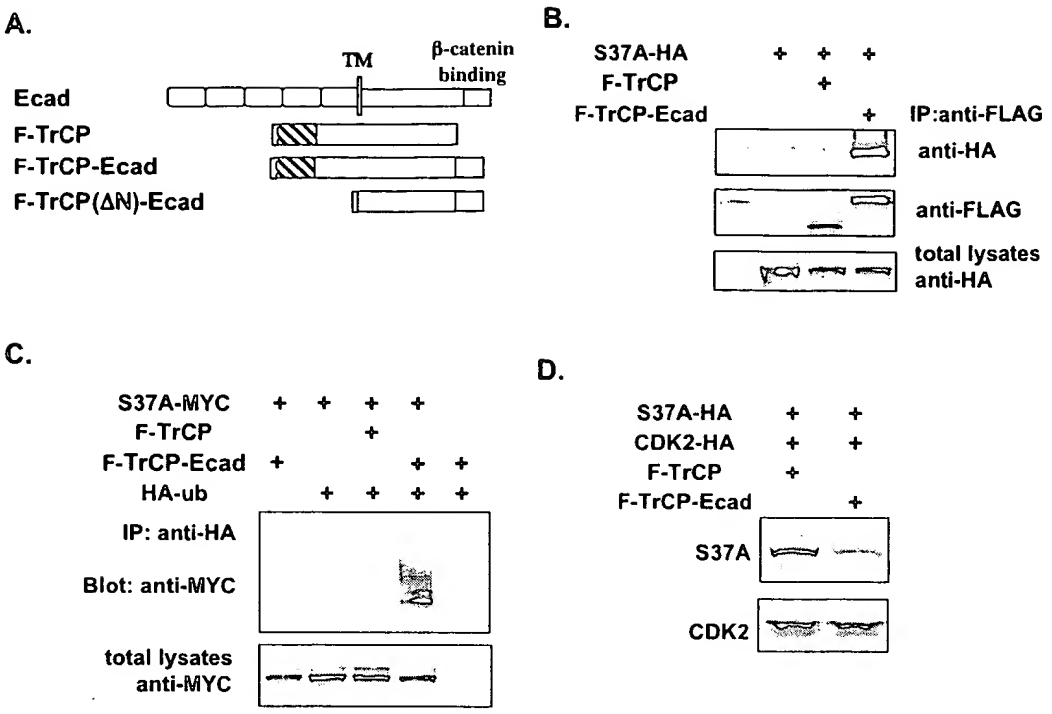

Figure 1

Engineering a βTrCP-E-cadherin chimera that targets oncogenic β-catenin for degradation. **A.** Schematic diagrams of F-TrCP-Ecad chimeras used in this study. The β-catenin binding domain (amino acids 794–883) of E-cadherin (Ecad) was fused to the C-terminus of FLAG-tagged βTrCP to form F-TrCP-Ecad. The N-terminus of βTrCP (amino acid 1–297) was deleted from F-TrCP-Ecad to make F-TrCP(ΔN)-Ecad. The transmembrane (TM) region and the β-catenin binding domain in E-cadherin are indicated. The F box of βTrCP is hatched. The FLAG epitope fused at the N-termini of βTrCP, βTrCP-Ecad, or βTrCP(ΔN)-Ecad is indicated by a solid rectangle. **B.** F-TrCP-Ecad binds to β-catenin S37A *in vivo*. HA-tagged β-catenin S37A and FLAG-tagged βTrCP derivatives were co-expressed in 293 cells. Cells were treated with the proteasome inhibitor MG132 for 4 hours before harvesting. Cell lysates were immunoprecipitated with the anti-FLAG antibody, precipitates were resolved by a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunoblotted with the anti-HA antibody (top panel). Note that a nonspecific band that cross-reacted with the anti-HA antibody migrated slightly above β-catenin S37A in all lanes of this panel. The membrane was stripped and blotted with the anti-FLAG antibody (middle panel). The expression of β-catenin S37A in total cell lysates was examined by immunoblotting with the anti-HA antibody (bottom panel). **C.** F-TrCP-Ecad induces ubiquitination of β-catenin S37A *in vivo*. MYC-tagged β-catenin S37A, HA-tagged ubiquitin, and indicated βTrCP derivatives were co-expressed in 293 cells. Cells were treated with the proteasome inhibitor ALLN for 4 hours, and lysed in the denaturing buffer by boiling to disrupt non-covalent protein-protein interactions. Cell lysates were immunoprecipitated with the anti-HA antibody, and immunoprecipitates were resolved in SDS-PAGE and blotted with the anti-MYC antibody (top panel). The expression of β-catenin S37A in total cell lysates was determined by immunoblotting with the anti-MYC antibody (bottom panel). **D.** F-TrCP-Ecad reduces the steady state levels of β-catenin. HA-tagged β-catenin and CDK2 were co-expressed with indicated βTrCP derivatives in 293 cells. Total cell lysates were resolved by SDS-PAGE, and immunoblotted with the anti-HA antibody.

intracellular domain of E-cadherin binds to the armadillo repeats of β-catenin in a phosphorylation-independent manner.

Next, we tested whether binding between F-TrCP-Ecad and β-catenin S37A could induce ubiquitination of β-catenin S37A. 293 cells were cotransfected with plasmids encoding HA-tagged ubiquitin and MYC-tagged β-catenin S37A. Cell lysates were immunoprecipitated with the anti-HA antibody under conditions that denature proteins in cell lysates, and probed with an anti-MYC monoclonal antibody against MYC-tagged β-catenin. As seen in Fig. 1C, β-catenin S37A was ubiquitinated only in the presence of F-TrCP-Ecad, but not F-TrCP.

To examine whether ectopic expression of F-TrCP-Ecad affects degradation of β-catenin S37A, plasmids encoding F-TrCP-Ecad and HA-tagged β-catenin S37A were cotransfected into 293 cells. A plasmid encoding HA-tagged CDK2 was also included in the transfection as an internal control. Consistent with the association between F-TrCP-Ecad and β-catenin S37A, co-expression of F-TrCP-Ecad significantly reduced the steady state level of β-catenin S37A, but not that of the control CDK2 (Fig. 1D). Therefore, the engineered F-TrCP-Ecad can recapture the "non-degradable" β-catenin S37A, and promote its ubiquitination and degradation.

We further tested the effect of F-TrCP-Ecad on the transcriptional activity of β-catenin. β-catenin S37A was transiently expressed in 293 cells with TOP-FLASH, a luciferase reporter that contains multiple copies of TCF binding sites and serves as a readout for β-catenin signaling activity [18]. As shown in Fig. 2A, co-expression of F-TrCP-Ecad dramatically decreased the transcriptional response to β-catenin S37A from the TOP-FLASH reporter. Two other β-catenin targeting peptides, which constitute the N-terminal domains of human TCF4 or *Xenopus* TCF3, were also fused to βTrCP, and demonstrated similar inhibitory effects on β-catenin signaling as F-TrCP-Ecad (data not shown).

The F box is critical for the ubiquitin-protein ligase activity of βTrCP. To investigate whether βTrCP-mediated β-catenin degradation is required for the attenuation of β-catenin signaling, an N-terminal region of βTrCP, including the F box, was deleted from F-TrCP-Ecad to form F-TrCP(ΔN)-Ecad, and tested for its effect on the transcriptional activity of β-catenin. Deletion of the N-terminal region from βTrCP resulted in a complete loss of inhibition of β-catenin signaling (Figure 2A), although F-TrCP(ΔN)-Ecad and F-TrCP-Ecad bound to β-catenin to the similar extent (Figure 2B). This finding suggests that the inhibitory effect of F-TrCP-Ecad on β-catenin signaling is dependent on targeted degradation of β-catenin by the engineered F-TrCP-Ecad ubiquitin-protein ligase.

There are two subpopulations of β-catenin inside cells, the nuclear/cytosolic pool and the membrane pool. Only the nuclear/cytosolic pool of β-catenin is subjected to direct regulation by Wnt signaling. The nuclear β-catenin binds to TCF/LEF transcriptional factors and activates the expression of Wnt target genes. The membrane β-catenin bridges the interaction between α-catenin and E-cadherin, and links the E-cadherin complex to the actin cytoskeleton.

We tested the effect of F-TrCP-Ecad induction on the levels of the membrane and cytosolic β-catenin in the DLD1 colon cancer cells. β-catenin signaling is deregulated in DLD1 cells due to inactivation mutations of APC, resulting in the accumulation of unphosphorylated β-catenin resistant to βTrCP-mediated proteolysis. A stable DLD1-F-TrCP-Ecad cell line was constructed in which the engineered F-TrCP-Ecad was expressed under the tetracycline-repressible promoter [19]. The expression of F-TrCP-Ecad was induced when DLD1-F-TrCP-Ecad cells were cultured in the absence of doxycycline (Dox), a tetracycline analogue (Fig. 3C). To test whether induction of F-TrCP-Ecad affects the steady state levels of the membrane and cytosolic β-catenin, DLD1-F-TrCP-Ecad cells were grown in the presence or the absence of Dox, and fractionated into the cytosolic pool (S100) and the membrane pool (P100) by centrifugation. As seen in Fig. 3A, induction of F-TrCP-Ecad expression reduced the steady state level of cytosolic β-catenin, while it had no effect on the level of the membrane-associated β-catenin. In contrast, Dox treatment of the parental DLD1 cells had no effect on the steady state level of cytosolic β-catenin (data not shown). Therefore, F-TrCP-Ecad preferentially targets soluble nuclear/cytosolic β-catenin for degradation, sparing the membrane-associated fraction, which is tightly associated with endogenous membrane E-cadherin.

The signaling activity of β-catenin was further assessed in response to induced expression of F-TrCP-Ecad. DLD1-F-TrCP-Ecad cells were transiently transfected with TOP-FLASH and a control plasmid encoding *Renilla* luciferase, then cultured in the presence or the absence of Dox. As seen in Fig. 3B, induction of F-TrCP-Ecad reduced TOP-FLASH activity by 4-fold, indicating decreased β-catenin signaling activity.

c-myc has been previously identified as a β-catenin target gene in colon cancer cells [5]. We asked whether c-myc expression was affected by down-regulation of β-catenin signaling. Indeed, induction of F-TrCP-Ecad by Dox withdrawal decreased the concentration of c-myc oncoprotein (Fig. 3C).

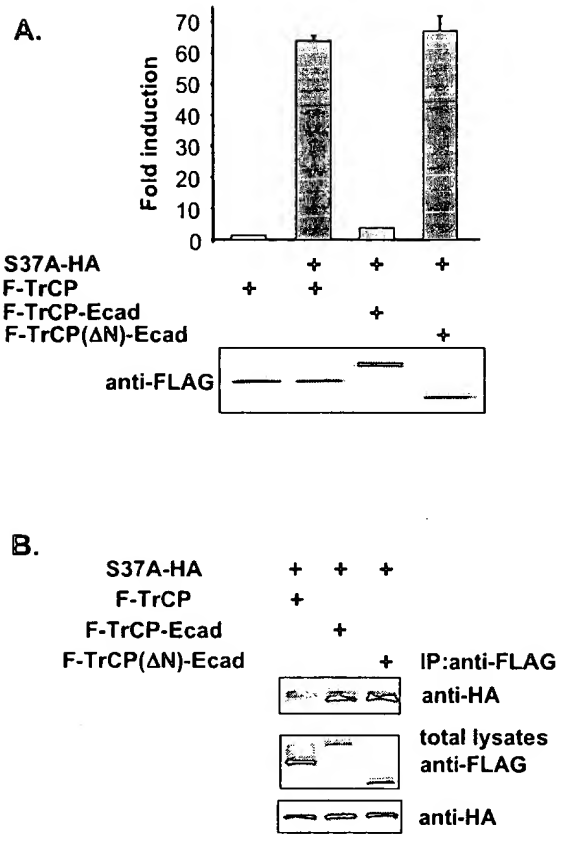

Figure 2

F-TrCP-Ecad inhibits the transcriptional activity of β-catenin S37A. **A.** 293 cells were co-transfected with a β-catenin S37A expression plasmid, indicated βTrCP expression plasmids, TOP-FLASH, and a CMV-*Renilla* reporter. The luciferase activities were measured and normalized against the control *Renilla* activities (top panel). The luciferase activity of cells transfected with βTrCP was arbitrarily set to 1. The expression of βTrCP derivatives was determined by immunoblotting with the anti-FLAG antibody (bottom panel). **B.** F-TrCP-Ecad and F-TrCP(ΔN)-Ecad bind to β-catenin to the similar extent. HA-tagged β-catenin S37A and FLAG-tagged βTrCP derivatives were co-expressed in 293 cells. Cells were treated with the proteasome inhibitor MG132 for 4 hours before harvesting. Cell lysates were immunoprecipitated with the anti-FLAG antibody, precipitates were resolved by SDS-PAGE and immunoblotted with the anti-HA antibody (top panel). The expression of FLAG-tagged βTrCP derivatives and β-catenin S37A in total cell lysates was examined by immunoblotting with the anti-FLAG and anti-HA antibodies (middle and bottom panels).

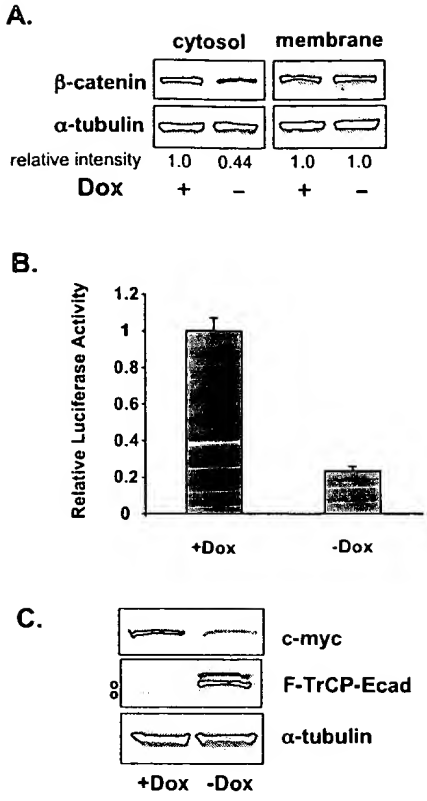

Figure 3

F-TrCP-Ecad promotes β-catenin degradation and inhibits Wnt signaling in DLD1 cells. **A.** Effects of F-TrCP-Ecad on the levels of cytosolic and membrane-associated β-catenin. A DLD1 inducible cell line was generated with the tetracycline (tet)-off system; these cells expressed F-TrCP-Ecad only in the absence of Dox. Cells were grown in the presence or absence of Dox for 5 days, and subjected to subcellular fractionation. Equal amounts of proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with the anti-β-catenin antibody. α-tubulin levels were determined by immunoblotting with the anti-α-tubulin antibody as an internal loading control. Quantified representation of immunoblot is shown at the bottom of each blot (relative intensity). **B.** F-TrCP-Ecad inhibits the transcriptional activity of β-catenin. DLD1-F-TrCP-Ecad cells were transfected with TOP-FLASH and a CMV-*Renilla* reporter, and cultured in the presence or absence of Dox. The luciferase activity of cells grown in the presence of Dox was arbitrarily set to 1. **C.** F-TrCP-Ecad induction inhibits c-MYC expression. DLD1-F-TrCP-Ecad cells were cultured in the presence or absence of Dox for four days. The expression level of c-myc and F-TrCP-Ecad were determined by immunoblotting with the anti-MYC (C-19) and the anti-FLAG antibodies (top and middle panels). Equal loadings were confirmed by immunoblotting with the anti-α-tubulin antibody (bottom panel). 'o' indicates two unknown species immuno-reactive with the anti-FLAG antibody, which migrated right below βTrCP-Ecad of the middle panel.

To study the effect of F-TrCP-Ecad induction on cell proliferation, we first measured the growth rate of DLD1-F-TrCP-Ecad cells in the presence or absence of Dox. A reduced rate of cell growth was observed when cells were cultured in the absence of Dox (Fig. 4A). This effect of F-TrCP-Ecad on cell proliferation was not a result of increased cell death, as determined by trypan blue staining (data not shown). A clonogenic assay was performed, in which DLD1-F-TrCP-Ecad cells were plated at a low density (2,000 cells/10 cm plate) and cultured in the presence or absence of Dox. Induction of F-TrCP-Ecad expression dramatically reduced the colony forming capability of DLD1 cells (Fig. 4B).

The effect of targeted-degradation of β-catenin on the tumorigenicity of DLD1 cells was examined by subcutaneous injection of DLD1-F-TrCP-Ecad cells into nude mice. Remarkably, nude mice fed with Dox-free food exhibited significant attenuation of tumor growth compared with those treated with Dox, indicating a requirement for maintenance of β-catenin levels for the tumorigenicity of DLD1 cells *in vivo* (Fig. 4C).

Collectively, these results provide strong evidence that induced expression of the engineered F-TrCP-Ecad ubiquitin-protein ligase downregulated β-catenin levels and significantly blocked Wnt signaling in and tumorigenicity of a colon cancer cell.

Discussion

The stability of β-catenin is tightly regulated in normal cells: β-catenin is constitutively phosphorylated by GSK3, which triggers its recognition and degradation by the $SCF^{\beta TrCP}$ ubiquitination machinery. In tumor cells with aberrant activation of the Wnt signaling pathway, β-catenin is no longer phosphorylated due to mutations in β-catenin itself or upstream elements that are critical for its phosphorylation, and thus escapes degradation. We have designed a protein knockdown strategy to recapture non-phosphorylated cytoplasmic β-catenin and to target it to the SCF machinery for degradation. This knockdown strategy can degrade both mutant β-catenin lacking N-terminal phosphorylation sites and wild-type β-catenin stabilized by APC mutations, and it preferentially targets cytoplasmic versus membrane β-catenin. Finally, we show that degradation of β-catenin results in inhibition of colorectal tumor cell growth both *in vitro* and *in vivo*.

β-catenin has both membrane and nuclear functions. To inhibit β-catenin signaling, one needs to specifically block the nuclear function of β-catenin while leaving the membrane function of β-catenin intact. Methods such as targeted gene knockout mutagenesis, RNA interference, or inhibition with antisense oligonucleotides will eliminate both the nuclear and the membrane functions of β-catenin. The nuclear function of β-catenin can be blocked by enhancing the degradation of cytosolic β-catenin with excess Axin [20,21] or APC [22,23]. However, neither Axin nor APC can induce the degradation of β-catenin mutants lacking N-terminal phosphorylation sites, and Axin has an apoptotic activity that appears to be independent of its effects on the Wnt pathway [24]. Another way to block the nuclear function of β-catenin specifically is to overexpress a dominant negative form of TCF; this approach has proved to be valuable for studying the role of the β-catenin in tumorigenesis [25]. However, there is evidence that at least some signaling functions of β-catenin might not be mediated by TCF. For example, activation of WISP-1 by Wnt and β-catenin appears to be TCF-independent [26]. Recently, it has also been shown that Pitx2 can recruit β-catenin to the promoter of Cyclin D2, and activate the transcription of Cyclin D2 [27].

The F-TrCP-Ecad chimera that we have engineered decreases the levels of cytosolic and presumably nuclear β-catenin, but has no obvious effect on the levels of membrane β-catenin. βTrCP has a short half-life [28](J.Z. and P.Z., unpublished results), so the steady state level of F-TrCP-Ecad in cells is likely low. It is possible that β-catenin, when in complex with the membrane E-cadherin, is protected from the action of F-TrCP-Ecad. However, prolonged high level expression of F-TrCP-Ecad eventually affects the abundance of membrane β-catenin through affecting the equilibrium between the two pools of β-catenin (J.Z. and P.Z., unpublished results).

In this study, we have found that down-regulation of β-catenin signaling in DLD1 cells reduces c-myc expression levels and impairs cell growth (Fig. 3 and 4) without causing obvious cell death (data not shown). Similarly, van de Wetering et al have shown that overexpression of a dominant-negative form of TCF4 in colon cancer cells causes growth arrest but not cell death [25]. Importantly, ectopic expression of *c-myc* can reverse the growth arrest induced by dominant-negative TCF4, demonstrating that c-myc is a crucial mediator of β-catenin oncogenic activity [25].

We have shown that the tumorigenicity of DLD1 cells appears to be dependent on the concentration of cytoplasmic β-catenin. However, this may not be true for all colon cancer cells with stabilized β-catenin. Using somatic cell gene targeting to disrupt either wild-type or mutant form of β-catenin, it has been shown that elevated β-catenin is not essential for the growth of HCT116 cells [29,30], but is required for the growth of DLD1 and SW48 cells [30]. Presumably, additional mutations in HCT116 cells override the need for an oncogenic signal mediated by β-catenin.

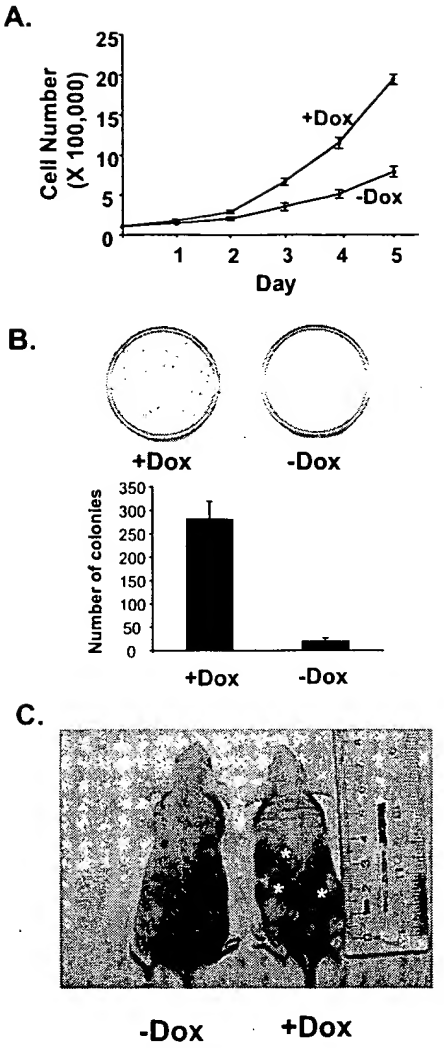

Figure 4
F-TrCP-Ecad inhibits clonogenic and tumorigenic potential of DLD1 cells. **A.** βTrCP-Ecad inhibits the growth rate of DLD1 cells. DLD1-F-TrCP-Ecad cells were grown in the presence or the absence of Dox for five days. The numbers of cells were counted daily in triplicates. **B.** Induction of F-TrCP-Ecad decreases the colony forming ability of DLD1 cells. DLD1-F-TrCP-Ecad cells were diluted, plated at a low density, and cultured in the presence or the absence of Dox for 14 days. Plates were photographed (top panel) and the number of colonies was counted (bottom panel). The experiment was performed in triplicates **C.** F-TrCP-Ecad induction inhibits tumorigenicity of DLD1 cells in nude mice. 2 × 10^6 DLD1-F-TrCP-Ecad cells were injected subcutaneously in nude mice. Each mouse received three injections. Nude mice were fed with either regular or Dox-containing food, and photographed three weeks after injection. In this observation period, six of six injections gave rise to tumors (indicated with asterisks) on mice fed with Dox-impregnated food, while none of the six injections generated tumor on mice fed with regular food.

Cancer stems from the step-wise accumulation of genetic changes. In simplified mouse models for cancer, continued activity of an initiating oncogene is usually required for tumor growth [31]. However, one cancer-causing gene important for tumor initiation might also become dispensable for tumor maintenance due to accumulation of additional mutations when the tumor progresses to an advanced stage [32]. Therefore, it is necessary to establish whether an oncoprotein in a late stage tumor is still a valid molecular target for the development of pharmacological inhibitors. The protein knockdown system provides a unique strategy for assessing the contribution of each oncoprotein to the tumorigenic phenotype of a late stage tumor as well as a novel approach for targeted tumor therapy.

Conclusions

We have developed a protein knockdown strategy to degrade unphosphorylated or mutated β-catenin, and demonstrated that suppression of β-catenin inhibits the neoplastic growth of colorectal tumor cells.

Methods

Cell culture

The 293 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. A tetracycline (tet)-off system was used for inducible expression of F-TrCP-Ecad protein. In this system, the repression of gene expression was achieved by maintaining cells in the presence of a tetracycline analog, doxycycline (Dox) and the induction of gene expression was achieved by Dox withdrawal. The DLD1-tTA cell line was a generous gift of Bert Vogelstein [19], which expresses tetracycline-suppressible transactivator (tTA)-IRES-neo under control of the CMV promoter. The DLD1-tTA cells were maintained in McCoy's 5A, 10% fetal bovine serum, 400 μg/ml G418, and 20 ng/ml Dox (ICN). The F-TrCP-Ecad expression construct was generated by cloning F-TrCP-Ecad into pBI-MCS-EGFP [19]. The construct was linearized and cotransfected with pTK-hygro (Clontech) into DLD1-tTA cells. Single colonies were obtained by limiting dilution with medium containing 400 μg/ml G418, 250 μg/ml hygromycin B (Clontech), and 20 ng/ml of Dox for three weeks. Clones with homogenous GFP induction were subjected to immunoblot analysis for the expression of F-TrCP-Ecad with anti-FLAG antibodies (Sigma). 293 and DLD1 cells were transfected with a calcium phosphate transfection kit (Stratagene).

Luciferase Reporter assay

Cells were split into 12-well plates 24 hours before transfection. Each well received 0.2 μg TOP-FLASH, 0.02 μg CMV-*Renilla* expression plasmid, together with 0.5 μg indicated plasmids. Thirty-six hour after transfection, cells were lysed and luciferase assays were performed with the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions. The luciferase activity was normalized with the *Renilla* activity.

Co-immunoprecipitation and immunoblotting assay

For co-immunoprecipitation experiments cells were lysed in EBC buffer (50 mM Tris, PH 7.6, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, and protease inhibitors). Cell lysates were clarified by centrifugation at 13,000 rpm for 15 minutes at 4°C. For immunoprecipitation, the cell lysates were incubated with 1 μg of appropriate antibodies at 4°C for 1 to 2 hours. The immunocomplexes were collected with Protein A or Protein G agarose beads, and washed five times with lysis buffer. The bound proteins were eluted with Laemmli sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature, and incubated with appropriate primary antibodies for 1 hour. Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibodies for 1 hour. Membranes were washed extensively with TBST and developed with an ECL kit (Amersham). Protein bands were semiquantitated by densitometry. Commercial antibodies used in this study include anti-FLAG (M2) and anti-α-tubulin monoclonal antibodies (Sigma), anti-HA (HA.11) monoclonal antibody (Covance), anti-β-catenin monoclonal antibody (Transduction Laboratories), anti-MYC (9E10) monoclonal antibody and anti-MYC (C-19) polyclonal antibody (Santa Cruz).

Cell fractionation assay

DLD1-F-TrCP-Ecad cells were grown in medium with or without Dox for 5 days before being harvested. Cells were washed and scraped on ice into TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 2 mM DTT, protease inhibitors). Cells were homogenized with 30 strokes in a dounce homogenizer, and the nuclei were removed by low speed centrifugation. The post-nuclear supernatants were spun at 100,000 g for 90 min at 4°C to generate a supernatant, or cytosolic, fraction and membrane-rich pellet fraction. Samples normalized for protein content were analyzed by SDS-PAGE.

Cell growth and colony formation assay

For growth curve analysis, cells (10^5) were seeded into a well of 6-well plates in the presence or the absence of 20 ng/ml Dox. Each day of 5 days, cells were counted using a hemocytometer after trypsinization. For colony formation assay, cells were plated at 2,000 cells/10 cm plate. Cells

were grown in the presence or the absence of Dox for 14 days and stained with 0.05% crystal violet (Sigma).

In vivo *Ubiquitination assay*

293 cells were transfected with a HA-tagged ubiquitin expression construct together with indicated plasmids. Thirty-six hours after transfection, cells were treated with the proteosomal inhibitor N-acetyl-leucinyl-leucinyl-norleucinyl-H (ALLN) for four hours. Cells were then harvested and lysed in 1% SDS lysis buffer (50 mM Tris-HCl pH7.5, 0.5 mM EDTA, 1% SDS, 1 mM DTT, protease inhibitors). Cell lysates were sonicated, heated at 80°C for 30 minutes, and clarified by centrifugation. Supernatants were diluted 10 times in Triton X-100 lysis buffer (50 mM Tri-HCl pH8.0, 150 mM NaCl, 1% Triton X-100, protease inhibitors). Cell lysates were incubated with anti-HA affinity matrix (Roche) overnight at 4°C. Immunoprecipitates were washed with Triton X-100 lysis buffer 5 times. The bound proteins were eluted with Laemmli sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting analysis.

Nude mouse assay

2×10^6 of DLD1-F-TrCP-Ecad cells were injected subcutaneously into 8-week-old athymic nu/nu (nude) mice (NCI). Animals were administered with regular or Dox-impregnated food pellets, and inspected for 3 weeks.

Authors' contributions

FC and PZ conceived the study and designed the experiments. FC, JZ, and WP performed the experiments. PZ and HV supervised the work. All authors read and approved the final manuscript.

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